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ANNOUNCEMENT

THE JOURNAL OF BIOLOGICAL CHEMISTRY enters upon its eighteenth volume with the present issue and the directors of the JOURNAL take this occasion to make the following announcement:

Up to the present time the work of both editing and publishing the JOURNAL has been done by the board of editors appointed by the directors. The rapid growth of the JOURNAL has been associated with a correspondingly large increase in the necessary labor of its production. After mature consideration it appeared that the objects for which the JOURNAL was established would be best served if arrangements could be made for transferring part of the business of publication to an efficient organization with existing facilities for the conduct of such work. The directors take pleasure in announcing that an arrangement has been consummated by which the Rockefeller Institute for Medical Research, an organization with which the founder of the JOURNAL was closely affiliated, will undertake for them the work of publishing the JOURNAL. At the same time the corporate ownership and editorial management of the JOURNAL remain unchanged. It is confidently believed that the future development and stability of the JOURNAL will be materially advanced by this arrangement.

With the present issue Dr. Donald D. Van Slyke joins the board of editors, and the directors are confident that this appointment will add to the strength of the JOURNAL.

No change is contemplated in the editorial policy of the JOURNAL, which will be conducted as heretofore solely with the object of providing a medium for the prompt publication of researches in every branch of biochemistry.

NUTRITIVE PROPERTIES OF PROTEINS OF THE MAIZE KERNEL.¹

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL,

WITH THE COÖPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

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and the Sheffield Laboratory of Physiological Chemistry in
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(Received for publication, March 24, 1914.)

The enormous production of maize in this country makes a knowledge of every factor concerning its nutritive value not only of scientific interest, but also of great economic importance. Experience in feeding the seeds of maize has led to the belief that, in comparison with many other available food stuffs, it is in some respects inadequate. To account for the nutritive deficiency various suggestions have been made, such as the low content of protein; the small percentage of ash; the relatively small proportion of calcium and large proportion of magnesium in the ash; and, recently, it has appeared possible that the peculiar chemical constitution of zein, the chief protein of this seed, may, in whole or in part, furnish an explanation. We have accordingly undertaken to determine the relative nutritive value of zein when fed in combination with other proteins of maize as well as of some other seeds and of milk and give in the following pages the results thus far obtained.

In order that the data here presented may be better understood in their relation to the practical use of maize products as food for animals or men it is necessary to call attention to the fact that this seed contains several distinct proteins which differ from one another in their solubilities and chemical constitution. The structural and nutritive deficiencies of zein are supplemented by these other proteins to a greater or less extent, and consequently

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

the statements relating to zein made in the following pages must not be understood to apply to the combination of proteins existing in the entire seed.

The most abundant protein of the maize kernel is zein which is soluble in relatively strong alcohol or dilute aqueous alkaline solutions, but insoluble in water or in solutions of neutral inorganic salts. The next most abundant is maize glutelin, insoluble in water, saline solutions or alcohol, but readily soluble in dilute sodium or potassium hydroxide solution. These two proteins contain about 72 per cent of the nitrogen of the entire maize kernel. About 22 per cent of the total nitrogen of the seed is soluble in saline solutions, such as 10 per cent sodium chloride solution, and probably belongs mostly to protein substances. From such extracts of corn meal Chittenden and Osborne² isolated three globulins, an albumin and a small amount of protein having the properties of proteose. Since the proportion of each of these is small, nothing has been learned of them beyond their ultimate composition and a few facts concerning their solubility. We have no information whatever as to their amino-acid make-up.

All of the foregoing proteins of the maize kernel are soluble in dilute alkaline solutions, but such solutions do not extract all of the nitrogen from even very finely ground corn meal. Whether any, or all, of this undissolved nitrogen belongs to proteins enclosed in unruptured cells or to non-protein substances insoluble in the solvents mentioned is unknown. Owing to the difficulties encountered in extracting these different types of protein, and in separating them completely from one another, no accurate statement can be made of the proportions in which they exist in the maize kernel. Some years ago one of us attempted to estimate the proportions of these several proteins in a sample of yellow corn which contained 1.54 per cent of nitrogen. The results of this attempt can be summarized as follows:

	Per cent
Globulins, albumins, and "proteoses".....	0.45
Zein.....	5.00
Maize glutelin.....	3.15
Insoluble N \times 6.25.....	<u>1.03</u>
	9.63

² Chittenden and Osborne: *Amer. Chem. Journ.*, xiii, pp. 453-468, 529-552, 1891; and xiv, pp. 20-44, 1892.

Further experience has shown that the aggregate amount of the proteins soluble in dilute saline solutions, *i.e.*, of the globulins, albumins, and "proteoses," is probably much greater than indicated by these figures which were based on the weights of preparations of these proteins actually isolated from the seed.

A more recent attempt, in which we were assisted by L. I. Holdredge,³ showed that about one-fifth of the nitrogen of a sample of white corn, containing 2.33 per cent thereof, was soluble in 10 per cent potassium chloride solution. Since most of this nitrogen undoubtedly belongs to protein we feel convinced that the proportion of the proteins soluble in saline solutions in our earlier analyses was stated too low. The following table shows the proportion of nitrogen found in the different parts of the seed.

White corn dried at 110°: N = 2.33 per cent.

	PER CENT OF CORN	CONTAINING N	
		Per cent of part	Per cent of corn
Hulls and tipcaps.....	8.5	1.52	0.12*
Embryo.....	11.0	3.42	0.38
Endosperm.....	80.5	2.28	1.84
	100.0		2.34

*The hulls alone contained 0.03 per cent N.

N extracted in per cent of total N.

	WHOLE CORN	ENDO- SPERM	EMBRYO	HULLS AND TIPCABS
N soluble in 10 per cent KCl solution	22.0	7.8	77.2	not deter- mined
N soluble in 90 per cent alcohol....	41.0	50.0	2.0	
N soluble in 0.2 per cent KOH solu- tion.....	31.0	38.2	0.6	
N insoluble and loss.....	6.0	4.0	20.2	

³ Not published.

N extracted in per cent of corn and its parts.

	WHOLE CORN	ENDO- SPERM	EMBRYO	HULLS AND TIPCAPS
N soluble in 10 per cent KCl solution	0.51	0.14	0.29	
N soluble in 90 per cent alcohol....	0.96	0.92	0.01	
N soluble in 0.2 per cent KOH solu- tion.....	0.72	0.70	trace	
N insoluble and loss.....	0.14	0.08	0.08	
Total N in corn.....	2.33	1.84	0.38	0.12

N in: Endosperm, 1.84; Embryo, 0.38; Hulls and tipcaps, 0.12 = 2.34

N in the whole corn..... 2.33

	ENDOSPERM + EMBRYO		FOUND IN WHOLE CORN
N soluble in 10 per cent KCl solution...	0.14	0.29 = 0.43	0.51
N soluble in 90 per cent alcohol	0.92	0.01 = 0.93	0.96
N soluble in 0.2 per cent KOH solution.	0.70	trace = 0.70	0.72
N insoluble and loss	0.08	0.08 = 0.16	0.14
N in hulls and tipcaps		= 0.12	
Total N.....		2.34	2.33

Assuming, as is probably nearly correct, that all of the nitrogen of the corn belongs to protein containing 16 per cent N, we have the following:

	PER CENT OF CORN	PER CENT OF PROTEIN
Globulins + albumins + "proteoses".....	3.19	21.9
Zein.....	6.00	41.4
Maize glutelin.....	4.50	30.8
Insoluble in alkali.....	0.88	5.9
	14.57	100.0

These figures cannot be accepted as exactly representing the actual proportions of nitrogen soluble in the various solvents, for many, as yet unsurmountable difficulties render complete extractions and accurate determinations impossible. We believe, however, that they give a fairly good idea of the relative proportions of the different types of protein, and must serve until more exact data can be secured.

In so far as they cover the same ground, the preceding data agree well with those given by Hopkins, Smith and East,⁴ who found a similar distribution of nitrogen among these different parts of a sample of high nitrogen corn. From these figures we can conclude that a little less than one-half of the protein substance in the entire maize kernel is zein. Since maize glutelin, the next most abundant protein, has been shown to yield all of the amino-acids which zein lacks, and as it is probable that the remaining proteins likewise yield them, the amino-acid deficiencies of zein are thus more or less supplemented when the entire seed is fed.

In the feeding experiments described in this paper a product was used which we have designated "corn gluten." This was kindly prepared for us under the direction of Mr. H. C. Humphrey of the Corn Products Refining Company. In manufacturing corn starch the seeds are softened in water containing sulphurous acid and the contents of the cells of the endosperm are separated from the other parts of the seed by grinding under water in suitable mills and straining out the hulls, tipcaps and embryos on sieves. The starch grains and suspended protein thus set free from the cells of the endosperm, after passing through the sieve, are carried slowly by the flowing water over long troughs, upon the bottom of which nearly all the starch is deposited so that the material passing out of the trough consists of nearly one-half endosperm protein and one-half starch, endosperm cell walls and other insoluble carbohydrates, and some oil. This material was filter-pressed, dried at a low temperature and sent to us as a source from which to obtain zein and maize glutelin.

The following figures show the results of our partial analysis of this preparation of "corn gluten."

	<i>Per cent of the "corn gluten" dried at 110°</i>
Inorganic matter.....	0.15
Total nitrogen.....	7.19
Ether-soluble matter.....	5.25
N soluble in hot alcohol.....	4.99
N soluble in hot alcohol $\times 6.25$ = zein.....	31.25
Total N minus alcohol-soluble N = 2.20×6.25 = maize glutelin.....	13.75
Total protein.....	45.00

⁴ Hopkins, Smith and East: Illinois Agricultural Experiment Station. Bulletin 87, 1903.

Inasmuch as this material had been extracted with a large quantity of water it was very thoroughly freed from water-soluble proteins, and since the embryos were practically all removed intact very little of the protein in this "corn gluten" could have been derived from this part of the seed. Nearly all of the protein was consequently derived from the endosperm, and consisted chiefly of zein and "maize glutelin." By extracting with 10 per cent potassium chloride solution we removed only 4.8 per cent of the nitrogen, or 0.35 per cent of the "corn gluten," equal to 2.2 per cent of protein containing 16 per cent of nitrogen.

In this "corn gluten" the ratio of zein to "maize glutelin" is 100:44 which is very much higher than was found in our analysis of the endosperm meal of the high nitrogen corn, namely 100:74. Whether the losses of maize glutelin incident to the processes employed in making this preparation of corn gluten were greater than those of the zein, or the proportion of zein to maize glutelin in the high nitrogen corn was less than in the sample of low nitrogen yellow corn which we used for our earlier analysis, cannot be determined from any data at present available. It is to be noted that in our earlier analysis 52 per cent of the total nitrogen was soluble in alcohol, whereas in the high nitrogen white corn only 41 per cent was soluble therein. If such a difference actually exists it ought to have a pronounced influence on the food value of these two types of corn, especially when fed to growing animals, since, as will be shown in this paper, growth proceeds at a rate determined by the degree in which the amino-acid deficiencies of zein are quantitatively supplemented by the addition of other proteins. These considerations show how important it is to have abundant and exact data concerning the relative proportions of the different types of nitrogen in the seeds of the numerous varieties of corn.

In a previous paper⁵ we have pointed out that the problems of the dietary in efficiency of zein hinge upon the peculiarities of its amino-acid make-up. When zein serves as the sole source of nitrogen in the ration, nutritive failure inevitably results sooner or later. This is not due to the failure to digest the protein or absorb its cleavage products. Zein is commonly regarded as rather difficult to digest; but when properly hydrated it is fairly well

⁵ Osborne and Mendel: this *Journal*, xvii, p. 325, 1914.

utilized. Thus for albino rats, which served as subjects in all our experiments, the "coefficient of digestibility," or utilization, calculated in the conventional way, ranged from 71 to 84 per cent, in contrast to 92 per cent for lactalbumin and 90 per cent for casein or gliadin. Obviously the unfavorable nutritive result cannot be charged to a lack of absorption of the digestion derivatives of zein.

The compilation of the most recent data in reference to the quantities of different amino-acids obtainable from zein by hydrolysis is given in our earlier paper.⁶ This brings out the complete lack of glycocoll, lysine, and tryptophane among them, as well as the relatively small yield of arginine and histidine. The character of the nutritive failures with our zein foods, as exemplified in declining body-weight, is exhibited in Chart IV appended to the publication just referred to.

With respect to the specific significance of the individual missing amino-acids little need be said about *glycocoll*. The consensus of opinion and the weight of experimental evidence, particularly as exemplified in hippuric acid production in the body, indicate that it can be synthesized anew by mammals. Its absence from the diet can therefore be made good within the organism itself. *Tryptophane* is indispensable, as we have already pointed out.⁷ No better illustration of its unique rôle in maintenance could be afforded than is given by rat 1892 in Chart V in the paper just referred to in which the inevitable failure and decline in body-weight on zein food was shown to be checked for 111 days when tryptophane equivalent to 3 per cent of the protein is added to it.⁸ In this experiment, which is still in progress, the body-weight of the animal has been maintained unchanged for 154 days. The entire absence of growth on foods containing zein + tryptophane in this and comparable experiments already published, in which young rats are nevertheless adequately maintained, indicates clearly the pronounced difference between the amino-acid requirement in growth and in maintenance. Only when all the necessary amino-acids are furnished can new construction of tissue proceed. In the

⁶ This *Journal*, xvii, p. 325, 1914.

⁷ *Ibid.*

⁸ Further data and discussion bearing on this are given in this *Journal*, xvii, p. 325 *et seq.*, 1914.

case of zein, therefore, the missing lysine must be supplied before growth takes place. *Lysine* will not replace tryptophane in making maintenance possible, as is shown by the failure of rat 1900 in Chart VI of the earlier paper.⁹ The function of these two amino-acids is elucidated when both are fed along with zein-food and growth results. We have appended a few new feeding trials in this direction (although the subject was dealt with in the earlier report) because they show such surprising success and emphasize so strongly the dominant importance and individual part played by certain amino-acids in nutrition—and further because the newer experiments contain the innovation of having additions of histidine and arginine in which zein is relatively, though not absolutely, deficient. (See Chart I.)

The decline in body-weight experienced by animals on a diet containing zein alone, along with non-nitrogenous foodstuffs, can be stopped not only by suitable additions of lacking amino-acids, but also by supplementing the ration with other proteins. This is, of course, precisely what happens when the maize kernel itself is fed. The zein in the seed is accompanied by an approximately equal quantity of other proteins: maize glutelin, albumins, globulins, "proteose," etc. Regarding the chemical structure of the latter nothing is known at present, except that maize glutelin yields all of the familiar amino-acid derivatives of protein. The induction of growth on a diet containing maize glutelin as the sole protein is shown by rats 547 and 567, Chart II.

It is possible to secure satisfactory growth of young rats on diets in which zein forms a very considerable portion of the ration provided that other suitable proteins are supplied in addition, as shown in a recent paper¹⁰ and further in the appended Chart III of experiments in which the supplementary protein consisted of casein. The relative proportions of these proteins requisite to convert the inadequate zein ration into a growth-promoting food vary quite widely and are dependent on their comparative content of the amino-acids in which zein is entirely or partially deficient.

On a diet having a protein concentration of 18 per cent of the solids of the ration, for example, growth may be slower than normal

⁹ Osborne and Mendel; this *Journal*, xvii, p. 325, 1914.

¹⁰ *Ibid.*

when the relative proportion of the supplementary protein added to the zein is smaller than that indicated in the satisfactory growth just referred to. The retardation becomes more conspicuous in each case with the proportionate decrease in the content of adequate protein. This is shown in Chart VIII already published.¹¹ A protein deficient in lysine, as is gliadin, fails to promote growth in any degree. (See Chart I¹²) If, as in this case, it contains sufficient tryptophane, it may suffice for maintenance alone. The rate of growth on otherwise suitable diets of zein containing additions of other proteins is therefore primarily dependent on the quantitative amino-acid make-up of the supplementary protein. The significance of this has already been emphasized in an earlier paper.¹³ The relative deficiency of the zein + casein combination in tryptophane there described in Chart VIII is exemplified by the accelerated growth which resulted on the addition of tryptophane. In the zein + edestin combination the shortage involves the lysine factor and can be made good by supplying this compound. (See Chart III of the earlier paper.)

In Chart IV are shown additional experiments in which zein has been supplemented by maize glutelin, vetch legumin, and phaseolin respectively. The body-weight of the animals was maintained in every case, and slow growth was secured in some instances, *e.g.* with a food containing equal parts of zein and maize glutelin. Foods in which the protein was either zein or phaseolin alone invariably proved inadequate for maintenance. When equal parts of both these proteins were present in the diet the animal was maintained, but growth was not secured. (See Chart IV.) Inasmuch as phaseolin is not entirely deficient in any of the amino-acids known to be yielded by proteins its ability to supplement zein so as to produce maintenance can be understood. No growth, however, has been secured. The failure of phaseolin alone to suffice for either maintenance or growth remains to be explained.

It is not necessary to use pure zein in order to exemplify the nutritive shortcomings of the chief protein of maize. The "corn gluten," to which reference has already been made on page 5 contains sufficient maize glutelin to permit of maintenance on

¹¹ This *Journal*, xvii, p. 325, 1914.

¹² *Ibid.*

¹³ *Ibid.*

this ration. More than very slow growth is impossible, however. (See Chart V.) Additions of other proteins to "corn gluten" have been made. The outcome is shown in Chart VI in which growth has resulted in accord with what one might expect from the amino-acid make-up of the proteins added. Lactalbumin, rich in both tryptophane and lysine, is a most efficient adjuvant; casein or edestin must be added in far larger proportion to accomplish results approaching those of the milk albumin.

The foregoing experiences bring into new light certain problems related to the economy of foods and commercial fodders. Corn forms the cheapest basis for the feeding of farm animals in food production. Inasmuch as the rate of growth is limited by hereditary, rather than nutritive, conditions, it is futile to furnish more energy, and particularly more protein, than is essential for normal development. An inadequate, but cheap protein, can be supplemented advantageously by one which supplies the needed factors, *i.e.*, amino-acids. The relative economy of these additions of supplementary proteins to an inefficient but inexpensive ration depends not only on their quantity but likewise on their amino-acid make-up. A very small addition of a protein like lactalbumin may be far more advantageous, when the cost per unit of gain is considered, than larger amounts of cheaper proteins which supplement the amino-acid deficiency of the standard diet less perfectly. It is perhaps not too utopian to expect that the day may come when amino-acid concentrates may serve to render perfect the mixtures of proteins in a fodder like maize or its commercial by-products.

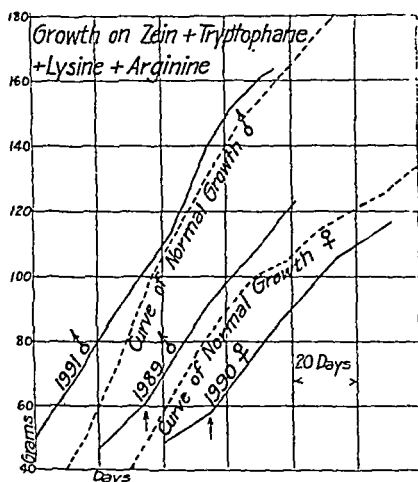


CHART I. GROWTH ON FOODS CONTAINING ZEIN + TRYPTOPHANE + LYSINE + ARGININE. For rats 1989 and 1990 at the periods indicated by the arrows *histidine* was added to the diet.

The food contained:

	Without histidine grams	With histidine grams
Zein.....	16.65	16.47
Tryptophane.....	0.54	0.54
Lysine.....	0.54	0.54
Arginine.....	0.27	0.27
Histidine.....	0.00	0.18
Protein-free milk.....	28.00	28.00
Starch.....	26.34	26.15
Butter-fat.....	18.00	18.00
Lard.....	9.00	9.00
Water.....	15.00	15.00

At present we attach no importance to the slight increase in the rate of growth shown by rats 1989 and 1990 after the addition of histidine to the diet, inasmuch as rat 1991 made quite as rapid growth without this amino-acid addition.

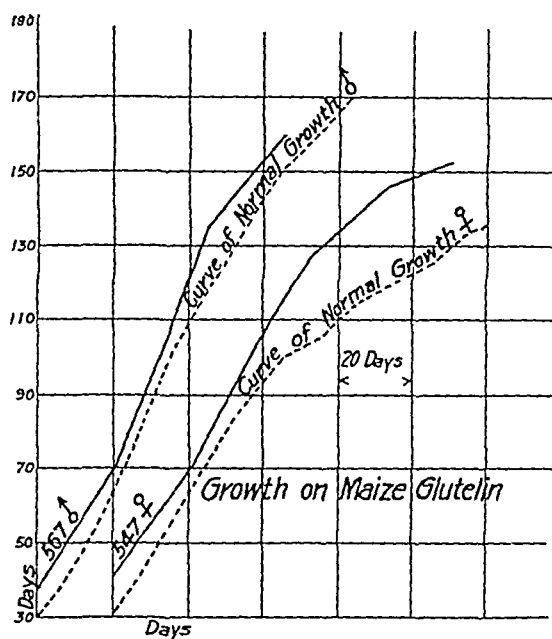


CHART II. GROWTH ON FOOD CONTAINING MAIZE GLUTELIN AS ITS SOLE PROTEIN.

The food contained:

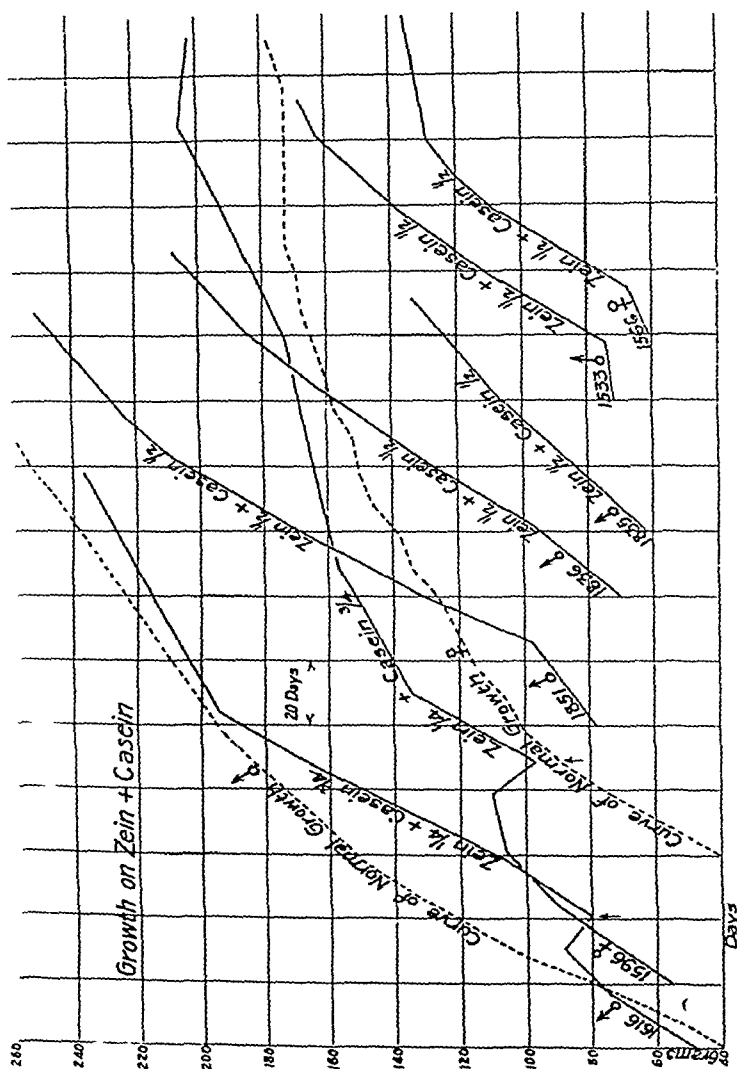
	Per cent
Maize glutelin.....	18
Protein-free milk.....	23
Starch.....	28
Lard.....	26

CHART III. (P. 13). EFFECT ON GROWTH OF REPLACING ZEIN WITH DIFFERENT PROPORTIONS OF CASEIN.

The foods contained:

	1616, 1596 grams	1851, 1836, 1835, 1533, 1566 grams
Zein.....	4.5	9
Casein.....	13.5	9
Protein-free milk.....	28.0	28
Starch.....	28.5	28
Butter-fat }	25.5	26
Lard }		
Water.....	3.8	7.5

Rats 1533 and 1566 received no butter-fat or other growth-promoting fat in the diet. For rats 1616 and 1596 butter-fat was added at the points indicated by the arrows, when the failure of growth already described in earlier papers (this *Journal*, xvi, p. 423, 1913) manifested itself.



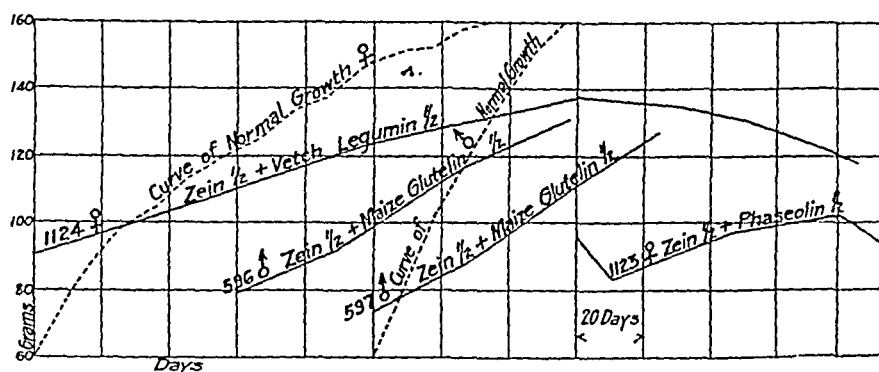


CHART IV. COMPARATIVE EFFECT OF MAIZE GLUTELIN, PHASEOLIN, AND VETCH LEGUMIN IN PREVENTING THE NUTRITIVE DECLINE CHARACTERISTIC OF ZEIN FOOD.

The foods consisted of:

	1124	596 597	1123
	grams	grams	grams
Zein.....	9.0	9	9.0
Maize glutelin.....	0.0	9	0.0
Phaseolin.....	0.0	0	9.0
Vetch legumin.....	9.0	0	0.0
Protein-free milk.....	23.0	23	23.0
Starch.....	27.5	24	27.5
Lard.....	26.5	30	26.5
Water.....	7.5	10	7.5

Although the proportion of the supplementary proteins used is alike in these experiments, maize glutelin is far more efficient in promoting growth than are the leguminous proteins here tested. It is interesting to note the maintenance of rat 1123 on a diet containing a mixture of proteins, either of which alone is inadequate for maintenance.

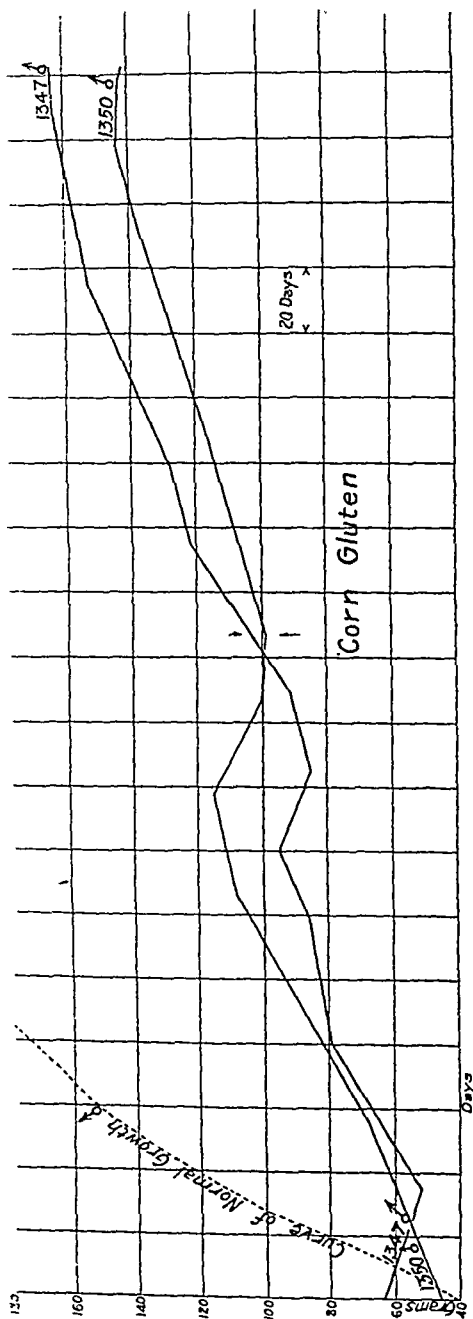


CHART V. MAINTENANCE AND SLOW GROWTH ON FOOD CONTAINING "CORN GLUTEN."

The food consisted of:

	per cent
"Corn gluten" (see page 5).....	38
Protein-free milk.....	28
Starch.....	2
Lard.....	32

Butter-fat, which has been found to promote growth was added to replace 18 per cent of lard, at the periods indicated by the arrows.

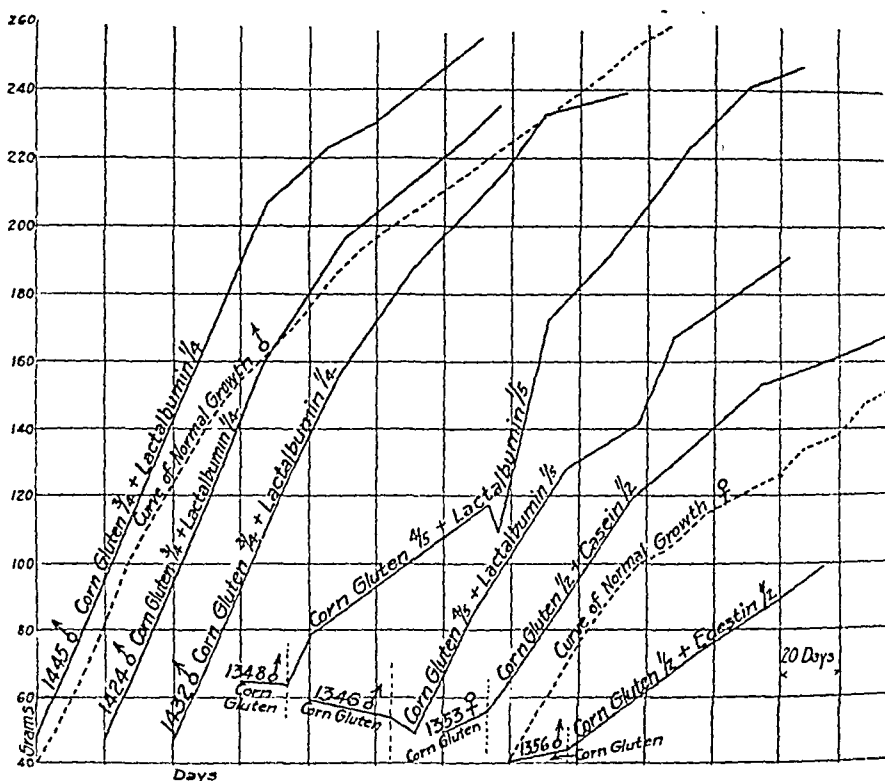


CHART VI. EFFECT OF SUPPLEMENTING "CORN GLUTEN" WITH PROTEINS ADEQUATE FOR GROWTH.

The food consisted of:

	1445	1348		
	1424	1346	1353	1356
	1432			
	per cent	per cent	per cent	per cent
"Corn gluten" (See page 5).....	28.5	30.4	10.0	10
Lactalbumin.....	4.5	3.6	0.0	0
Casein.....	0.0	0.0	9.0	0
Edestin.....	0.0	0.0	0.0	9
Protein-free milk.....	28.0	28.0	28.0	28
Starch.....	8.5	7.2	17.5	16
Lard.....	30.5	30.8	26.5	28

The effect of corn gluten alone (see composition of food for Chart V) is shown in the earlier parts of the curves for rats 1348, 1346, 1353, and 1356. It will be noted that, for reasons explained in the text, the smaller supplementary portions of lactalbumin are even more effective than larger admixtures of casein and edestin in promoting growth. The rapid recovery of rat 1348 from the decline noted at the end of 77 days is due to the substitution of natural protein-free milk for the artificial protein-free milk IV described in this *Journal*, xv, p. 317, 1913.

THE COMPARATIVE CHEMISTRY OF MUSCLE: BETAINES FROM THE SCALLOP, PERIWINKLE¹ AND LAMPREY: CREATINE FROM THE LAMPREY.²

By D. WRIGHT WILSON.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Connecticut.)

(Received for publication, April 6, 1914.)

The qualitative examination of muscle extracts from various animals has led to the discovery of a large series of compounds the relationships of which have been contrasted in a general way in a previous paper.³ Since Chevreul,⁴ in 1835, discovered the presence of creatine in muscle, the knowledge of its occurrence has gradually been extended. It has not been isolated from an invertebrate, and Krukenberg⁵ showed many years ago that even the characteristic color reaction of Weyl could not be obtained with suitably treated extracts of these animals. All of the undisputed data available at present indicate that creatine is a constant constituent of vertebrates and is absent from invertebrates. Mellanby,⁶ in a comparative study of muscle creatine using Folin's colorimetric method, reported that the muscle of the lamprey, one of the lowest vertebrates available, contains about one-half as much of that compound as does the muscle

¹ Reported in brief before the Society for Experimental Biology and Medicine, May 21, 1913. See *Proceedings*, x, p. 164, 1913.

² Reported in brief before the Society for Experimental Biology and Medicine, October 15, 1913, by Wilson and Lyman. See *Proceedings*, xi, p. 22, 1913.

³ Wilson: this *Journal*, xvii, p. 385, 1914.

⁴ Chevreul: *Journ. de pharm.*, xxi, pp. 231-242, 1835.

⁵ Krukenberg: See v. Fürth, *Vergleichende chemische Physiologie der niederen Tiere*, Jena, 1903.

⁶ Mellanby: *Journ. of Physiol.*, xxxvi, pp. 447-487, 1903.

of the rabbit. The fact that color reactions for creatine (creatinine) have occasionally been obtained in unsatisfactory or uncharacteristic fashion with certain extracts of invertebrate muscles shows that no dependence can be placed upon such non-specific colorimetric identifications for this compound unless they are substantiated by the actual isolation of the latter. In the case of unstripped muscle of vertebrates, for example, it is by no means agreed that creatine actually occurs there; other derivatives may simulate Jaffé's reaction. It was thought desirable, therefore, to attempt the isolation of creatine from the extract of the muscle of the lamprey, the lowest form of vertebrate available. The actual identification of creatine, as described below, together with the lack of even a colorimetric indication of appreciable quantities of this compound in extracts of muscles of invertebrates completely confirms the idea of a fundamental difference between vertebrates and invertebrates in regard to the metabolism of creatine.

The compounds isolated from vertebrate muscle extracts appear to be, in general, more complex than those isolated from invertebrate extracts. Carnosine, a compound of histidine and alanine found in beef extract, is apparently replaced by the amino-acids, histidine, arginine and lysine or, indeed, by the monamino-acids like glycocoll, in the invertebrates. Recent quantitative studies have shown that, in the higher vertebrates, the carnosine and creatine present account for two-thirds of the extractive nitrogen⁷ whereas, in the invertebrates, the monamino-acid nitrogen may be over 50 per cent of the total extractive nitrogen.⁸ In contrast with the relatively complex methylated compounds like oblitine, carnitine, and myokynine found in the higher animals is the presence of betaine—trimethyl glycocoll—in the invertebrates and lower vertebrates. With respect to the presence of betaine and the absence of creatine, the invertebrates closely resemble plants. The literature on the occurrence of betaine will be found in a previous paper.⁹

⁷ v. Fürth and Schwarz: *Biochem. Zeitschr.*, xxx, pp. 413-432, 1910.

⁸ Wilson: *loc. cit.*

⁹ Wilson: *loc. cit.*; See also Suzuki et al.: *Journ. of the Coll. of Agric., Imperial University of Tokyo*, v, pp. 1-25, 1912; Joshimura and Kanai: *Zeitschr. f. physiol. Chem.*, lxxxviii, pp. 346-351, 1913.

EXPERIMENTAL.

Betaine from the scallop (Pecten irradians).

The fresh adductor muscles (2 kgm.) were hashed and extracted with several portions of hot water at about 70°-80°C. The protein was coagulated by acidification with acetic acid and boiling, and the filtrate concentrated on a water bath to a volume of about 3 liters. Two volumes of 95 per cent alcohol were added to precipitate the glycogen. The solution was allowed to stand over night and filtered. The alcoholic filtrate was concentrated under reduced pressure to about 2 liters and freed from residual albuminous material by the Kutscher procedure with tannin, barium hydroxide, lead oxide and hydrogen sulphide.¹⁰ The resulting solution was concentrated under reduced pressure, at about 30°-40°C. to a thick syrup. Glycocoll crystallized out on standing. The filtrate therefrom was diluted to 200 cc. and treated according to Kossel's method with silver nitrate and barium hydroxide. The filtrate from the silver-baryta precipitate was freed from silver and barium by sulphuric acid and hydrogen sulphide, concentrated to 300 cc. (under reduced pressure) and precipitated by phosphotungstic acid in the presence of 5 per cent sulphuric acid. The phosphotungstic acid precipitate was decomposed in the usual way and the resulting solution concentrated under reduced pressure. The addition of alcohol to the strongly alkaline solution precipitated 0.5 gram of betaine as small white crystals. Alcoholic hydrogen chloride was added to the mother liquor whereupon a white crystalline precipitate of betaine hydrochloride formed immediately. The precipitate was recrystallized from water and two fractions were obtained (I, 0.4 gram; II, 0.7 gram) both of which melted at 228°-230°C. with decomposition. The second fraction contained some potassium chloride. The crystals were dried at 100° and analyzed.

	Calculated for $C_2H_{11}NO_2 \cdot HCl$:	Found:	
		I	II,
N.....	9.12	10.05	8.1
Cl.....	23.09		23.2

A part of the first fraction was dissolved in hot 95 per cent alcohol and an alcoholic solution of platinic chloride was added. Small orange-yellow needle-like prisms were obtained which were recrystallized from hot 95 per cent alcohol and dried at 110°. The substance melted at 234°-235° with decomposition.

	Calculated for $(C_2H_{11}NO_2 \cdot HCl)_2PtCl_6$:	Found:
Pt.....	30.31	30.37

An alcoholic solution of picric acid was added to a water solution of the betaine hydrochloride. Small yellow needles of betaine picrate were obtained which melted at 180°.

¹⁰ Abderhalden: *Handbuch der biochemischen Arbeitsmethoden*, ii, p. 1044.

Betaine from the periwinkle (Sycotypus caniculatus).

The pedal muscles (8.3 kgm.) were hashed and treated as described for the scallop. Thirty grams of the free base were isolated. From this and the mother liquor, 50 grams of betaine hydrochloride were obtained. The substance melted at 229°-230° with decomposition. It was dried at 110° for analysis.

	Calculated for $C_3H_{11}NO_2 \cdot HCl$:	Found:
N.....	9.12	9.35
Cl.....	23.09	22.8

The platinum double salt melted at 234°-235° with decomposition. It was dried at 110° for analysis.

	Calculated for $(C_3H_{11}NO_2 \cdot HCl)_2PtCl_4$:	Found:
Pt.....	30.31	30.00

The picrate melted at 178° with decomposition.

Creatine and betaine from the lamprey (Petromyzon marinus).

The body muscles were treated like the muscles described above, except that the extract was not precipitated with alcohol prior to the treatment with tannin. An abundance of creatine and creatinine separated on long standing from the concentrated extract. After extraction with absolute alcohol and recrystallization, the creatine was found to be free from creatinine. It was dried at 100° and analyzed.

	Calculated for $C_4H_7N_3O_2$:	Found:
N.....	32.0	32.2 32.5

A portion was hydrolyzed and the creatinine estimated by Folin's colorimetric method.

Weight creatine used, 13.3 mgm. Weight creatinine estimated, 11.4 mgm. Weight calculated as creatine, 13.2 mgm.

From the silver-baryta filtrate, betaine was obtained mixed with a considerable quantity of potassium chloride. Difficulty was experienced in purifying the betaine on account of the small quantity present and only 0.4 gram was obtained in pure condition. It melted at 228°-230°C. with decomposition. It was dried at 110° for analysis.

	Calculated for $C_3H_{11}NO_2 \cdot HCl$:	Found:
N.....	9.12	9.61
Cl.....	23.09	23.31

The platinum double salt melted at 235°.

SUMMARY.

Betaine was isolated from the extracts of muscles from the scallop, periwinkle and lamprey.

Creatine was isolated from the extract of the muscle of the lamprey.

I wish to thank Prof. Lafayette B. Mendel for suggesting this work.

CERTAIN NEW PRINCIPLES CONCERNING THE MECHANISM OF HYPERGLYCAEMIA AND GLYCOSURIA.

By ALBERT A. EPSTEIN AND GEORGE BAEHR.

(From the Pathological Laboratory of the Mount Sinai Hospital, New York.)

(Received for publication, April 7, 1914.)

In normal animals even under constant experimental conditions the percentage of sugar in the circulating blood may vary to such an extent that often from a single examination of blood no positive conclusion as to the existence of a moderate grade of hyperglycaemia can be drawn. This is well illustrated in Table 1, column 5. In much of the experimental work upon the blood sugar, this fact has been left out of consideration and the average values obtained on a number of normal animals has usually been used as the criterion for judging the presence and the degree of hyperglycaemia.

In order to furnish more accurate controls in certain investigations upon glycosuria in which we have been engaged, it became necessary to determine the normal blood sugar value for each animal, prior to performing the experiment. We were immediately confronted with the fact first observed by Claud Bernard¹ and since by others, that the mere withdrawal of a small amount of blood from an animal was sufficient in itself to induce a definite increase in the percentage of the blood sugar as determined by subsequent examinations. This phenomenon, as illustrated in Table 1 seems to be without exception.

Those who have made this observation have attributed the rise in the percentile sugar content to one of two causes, namely the operative interference on the one hand and psychic influences on the other (*Schreckhyperglykemie* of Jacobsen,² *Fesselungsdiabetes*

¹ *Leçons sur le diabète*, p. 210.

² *Biochem. Zeitschr.*, li, p. 443, 1913.

of Boehm and Hoffmann³). Of late Bang⁴ has accepted the psychic influence as the chief factor in the causation of this phenomenon, although shortly before he was inclined to minimize its importance.

Although the importance of psychic influence cannot be denied, our experiments demonstrate conclusively that it can by no means be the sole factor concerned in the production of the hyperglycaemia following bleeding. Bang admits that by keeping a rabbit for some time in a laboratory environment and by repeated handling, the *Schreckhyperglykemie* may be reduced to a negligible amount. And yet in our own experiments when all necessary precautions to avoid frightening or hurting the animal were taken, simple bleeding invariably resulted in a definite increase in the percentage of the blood sugar.

The technique of the procedure consisted simply in bleeding a rabbit from an ear vein. The animals were usually in the laboratory for some days or weeks prior to experimentation, and were accustomed to being handled. They were starved for twelve to twenty-four hours before the experiment in order to avoid post-prandial fluctuations in the blood sugar content. Before and during the bleeding, the animals were always handled very gently and every effort was made to avoid any excitement. In the simple bleeding experiments (see Table 1), 10-31 cc. were withdrawn, followed in from 80 to 190 minutes by a second bleeding. The blood was immediately collected in 2 per cent HCl or in a 2 per cent solution of sodium fluoride, treated according to the procedure of Schenk and the sugar titrated according to Pavy's method.

In the second series of experiments (see Table 2), the blood withdrawn was immediately replaced by an equal volume of sterile physiological salt solution slowly administered intravenously. The amounts withdrawn and infused varied from 10 to 45 cc. From 100 to 135 minutes after the bleeding, the second bleeding was performed.

An argument advanced by some observers in favor of the predominating influence of the psychic element in such experimental hyperglycaemias is based upon the lack of relationship between the amount of blood drawn and the intensity of the hyperglycaemia. From our own figures we are inclined to view this lack of relationship as more apparent than real. For if one takes into consideration the size of each animal and estimates therefrom the probable

³ *Arch. f. d. ges. Physiol.*, xxiii, 1880.

⁴ *Der Blutzucker*, Wiesbaden, 1913.

total volume of its blood, the increase in the percentage of sugar is usually seen to bear a striking relationship to the proportion of blood left in circulation after the bleeding.

TABLE 1.

1 RABBIT	2 WEIGHT	3 FIRST HEMOR- RHAGE	4 SECOND HEMORRHAGE	5 TIME ELAPSED	6 SUGAR BEFORE EXP.	7 SUGAR AFTER EXP.	8 TOTAL SUGAR BEFORE EXP.	9 TOTAL SUGAR AFTER EXP.
	grams	cc.	cc.	minutes	per cent	per cent	mgm.	mgm.
1	1450	10	10	80	0.130	0.164	95	103
2	2100	10	10	120	0.130	0.154	137	146
3	1860	10	10	190	0.125	0.143	116	118
4	1800	20	10	120	0.125	0.182	113	127
5	1800	20	10	135	0.154	0.231	138	162
6	2150	26	10	108	0.166	0.234	178	190
7	2100	30	10	120	0.105	0.154	110	115
8	2800	31	20	113	0.118	0.183	165	199

For example, in experiment No. 7, the rabbit weighed 2100 grams; its total blood volume before the bleeding was computed at 105 cc. (5 per cent of the body-weight). The amount of blood drawn was 30 cc., in other words, 75 cc. of blood was left in circulation. The percentage of sugar in the blood as drawn was 0.105 per cent; two hours after the bleeding the sugar content rose to 0.154 per cent.⁵ By comparing the total volumes of blood in the animal before and after the bleeding, they are seen to bear the ratio to one another approximately as 3:2. On the other hand the percentages of sugar in the blood before and after the bleeding are to one another as 2:3. In other words, the percentage of sugar in the blood is inversely proportional to the blood volume.

⁵ The very marked hyperglycaemias following blood letting obtained by Lewandowsky (*Arch. f. Physiol.*, 1901, p. 365), 0.31 per cent, Anderson (*Biochem. Zeitschr.*, xii, p. 1, 1908), 0.37 per cent, and Erlandsen (*ibid.*, xxiii, p. 329, 1910), 0.31 per cent; is due to an exaggeration of the normal rise in the percentage of blood sugar after bleeding by other complicating factors such as psychic disturbances, operative interference, etc. These figures were obtained after bleeding from the carotid artery. Others who bled from an ear vein (Rose: *Arch. f. exp. Path. u. Pharm.*, 1, p. 15, 1903; Anderson, *loc. cit.*, exps. 12 and 13) obtained subsequent increases in blood sugar percentage which are comparable to those observed by us.

The significance of this observation is only fully appreciated if the total sugar content in the blood before and after the bleeding is computed. As seen by comparing the figures in columns 8 and 9, Table 1, the total sugar content of the blood one and a half to two hours after the bleeding tends to remain approximately what it was before the bleeding. In experiments 1, 2, 3, 4 and 7 the differences between the total blood sugar determinations before and after the bleeding are so small that they come fairly within the limits of error. Experiments 5, 6 and 8 show greater differences which may be due to a physiological overcompensation. But the uniformity of the results in the majority of the experiments could hardly be expected were the percentile rise purely of psychic origin.

In other words the hyperglycaemia following simple bleeding is relative, *i.e.*, percentile and not absolute. It is quite incomprehensible why this should be so, unless we assume that it is the result of a physiological effort on the part of the body to maintain the rate of sugar supply to the tissues at an approximately constant level.

This hypothesis is made still more plausible by the interesting observation, in which we have the support of Lewandowsky,⁶ Rose,⁷ Bang,⁸ and others, that in no instance does a glycosuria develop even in those animals in which the rise in the percentage of blood sugar following the bleeding was considerable. It is well known how relatively easy it is to produce a glycosuria in rabbits. One would therefore expect that the rise in the sugar concentration of the blood ought to produce a glycosuria. The significance of the absence of glycosuria under such conditions of hyperglycaemia will be discussed later.

In order to test the above mentioned hypothesis still further, we varied the experiments in so far that we replaced the blood withdrawn with physiological salt solution. If it be true that the total quantity of sugar circulating in the blood under normal conditions has a tendency to remain constant, then replacing the blood withdrawn by an equal quantity of normal saline solution should not ultimately lead to a change in the percentage concentration of the sugar.

⁶ *Loc. cit.*

⁷ *Loc. cit.*

⁸ *Loc. cit.*

TABLE 2.

1 RABBIT	2 WEIGHT	3 FIRST HEMORRHAGE	4 SALINE INFUSED	5 SECOND HEMORRHAGE	6 TIME ELAPSED	7 SUGAR BEFORE EXP.	8 SUGAR AFTER EXP.	9 TOTAL SUGAR BEFORE EXP.	10 TOTAL SUGAR AFTER EXP.
	grams	cc.	cc.	cc.	minutes	per cent	per cent	mgm.	mgm.
1	2550	10	10	10	130	0.200	0.200	254	254
2	1650	20	20	10	130	0.114	0.126	95	105
3	2050	28	28	11.5	123	0.142	0.150	146	154
4	2400	30	30	10	115	0.180	0.180	216	216
5	2050	36	36	10	112	0.154	0.150	159	154
6	3700	37	37	11.5	135	0.154	0.200	285	370
7	2800	45	45	31	110	0.110	0.118	154	165
8	3400	43	44	21	115	0.138	0.143	235	243
		21	20	21	100	0.143	0.138	243	235

The results obtained in these experiments are much more striking than in the previous series. In all the experiments but one the percentage concentration of the sugar two hours after the bleeding and infusion is about the same as that found before the bleeding. Even repeated bleeding followed by infusion did not result in any hyperglycaemia (Exp. 8), and two hours after the last bleeding both the percentage and the total blood sugar remained the same as before the beginning of the experiment.

It must be understood that the first effect of the saline infusion following the bleeding is of course a depression in the percentage of blood sugar. But this rapidly rises until, within one and a half to two hours, the previous normal level is attained. The second estimation (column 8) was therefore made one and a half to two hours after the bleeding. It might be argued that this rise in the sugar concentration was not due to an increased mobilization of sugar but rather to the fact that the diluting fluid left the blood vessels. That this was not the case was easily demonstrable by means of hemoglobin estimations, which showed that two hours after the bleeding and infusion the blood was just as dilute as immediately after the infusion. It was therefore justifiable to conclude that the blood volume had remained unchanged.

* The exception (Exp. 6) shows a hyperglycaemia after bleeding and infusion which is not only relative but absolute. No reason for the discrepancy, such as variation in technique, was apparent.

DISCUSSION.

Contrary to Bang's recently expressed opinion, the above observations would seem to demonstrate that the hyperglycaemia following simple bleeding is not merely psychic, but that it is a compensatory response on the part of the organism to keep the total blood sugar up to a level commensurate with the needs of the tissues. In view of the diminished blood volume this is accomplished by an increase in the concentration.

Similarly, if the total volume of fluid in circulation be not diminished, the blood withdrawn being immediately replaced by saline solution, the percentage concentration of the diluted blood rapidly rises (with occasional exceptions) to what it was before the experiment. In other words the total sugar in the circulation remains unchanged.

It would seem therefore that at least under such conditions as in the above experiments, an increase in the percentage of sugar in the blood is only indicative of a relative, that is to say percentile, but not of an absolute hyperglycaemia.

What is still more important is, that in view of the uniform absence of glycosuria under such conditions the experiments suggest very strongly that glycosuria is the result of an absolute hyperglycaemia and need not occur when the hyperglycaemia is only relative. This would help explain the presence in certain individuals of glycosuria without hyperglycaemia, and conversely the absence of glycosuria when hyperglycaemia is present.

It is possible that the above observations may account for some of the so-called examples of renal glycosuria. The diagnosis in human beings of a condition of increased permeability of the kidney for sugar, in other words of a renal glycosuria, rests entirely upon the absence of any demonstrable hyperglycaemia.¹⁰ The cases reported by Naunyn,¹¹ Luthje,¹² Bönninger,¹³ Frank¹⁴ and others are now generally admitted to be rather questionable. It is not altogether improbable in view of the above experiments

¹⁰ Bang: *Der Blutzucker*, p. 133.

¹¹ Naunyn: *Diabetes Mellitus*, 2 Aufl., 1906, p. 131.

¹² Luthje: *Münch. med. Wochenschr.*, 1901, No. 38.

¹³ Bönninger: *Deutsch. med. Wochenschr.*, 1903, p. 780.

¹⁴ Frank: *Verh. d. Kong. f. inn. Med.*, 1913, p. 166.

that the glycosurias which they described were dependent upon an increase in the total blood sugar and that the absence of any hyperglycaemia could be accounted for by the existence in these individuals of an abnormally large blood volume.¹⁵

In following up this line of thought, we would therefore suggest the advisability of studying blood volumes and computing therefrom the total blood sugar in cases of diabetes mellitus. For therein may lie the explanation for the disproportion between the degree of hyperglycaemia and the intensity of the glycosuria, which occurs so frequently in this condition.

¹⁵ This may also apply to those examples of so-called renal glycosuria first described by Novak, Porges and Strisower (*Wien. klin. Wochenschr.*, 1913) as occurring in pregnant women.

THE FORMATION OF AMINO- AND HYDROXY-ACIDS FROM GLYOXALS IN THE ANIMAL ORGANISM.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, April 8, 1914.)

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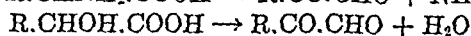
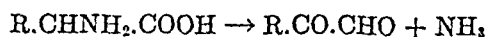
I. General introduction.

The possibility that substances belonging to the group of glyoxals may play a rôle in the intermediary metabolism of proteins and carbohydrates follows from a variety of observations which have been previously recorded.¹ The more essential points relating to these observations, which include experiments made both *in vitro* and in the living organism, may be summarized as follows:

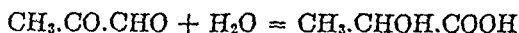
α -Amino and α -hydroxy-acids yield glyoxals when digested at

¹ This *Journal*, 1913 and 1914.

body temperature with substances such as *p*-nitrophenylhydrazine, which form insoluble derivatives with the glyoxals.



Glyoxals thus formed may be reconverted into α -hydroxy-acids by the action of widely distributed enzymes, glyoxalases. Methyl glyoxal, for example, yields lactic acid:

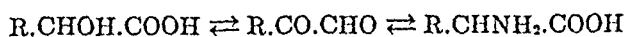


The action of glyoxalase is inhibited by a thermolabile substance or enzyme "antiglyoxalase" which had hitherto been found solely in the pancreas, and it appears probable that antiglyoxalase represents a mechanism for the regulation of the action of glyoxalase.

Methyl glyoxal may be readily formed *in vitro* from glucose and from lactic acid and alanine. Conversely when methyl glyoxal or *l*- or *d*-lactic acid² which are formed from it by the action of glyoxalase, or *d*- or *l*-alanine, are given to a dog rendered glycosuric by phlorhizin, they are all converted into glucose. Methyl glyoxal, therefore, may be regarded as a possible intermediary stage in the interconversion of glucose, lactic acid and alanine.

Finally it has been shown that a glyoxal, such as phenyl glyoxal, may yield the corresponding α -ketonic acid on perfusion through a surviving liver. The important biochemical relationships of the α -ketonic acids are well known from the work of Neubauer, Knoop, Embden and others.

By combining the results obtained from many different types of experiments, it appeared that α -amino- and α -hydroxy-acids might be regarded as being in equilibrium with the corresponding glyoxals and that the latter substances might be intermediate stages in the interconversion of amino- and hydroxy-acids. The relationship may be crudely represented as follows:



But hitherto, while we had demonstrated glyoxal formation from α -amino- and α -hydroxy-acids *in vitro* and had observed the production of hydroxy-acids from glyoxals both *in vitro* and *in*

² The experiments with *d*-lactic acid are due to Mandel and Lusk.

vivo, we had not determined the formation of amino-acids from glyoxals. We have, in the meantime, endeavored to observe this reaction both within the body and without. Our results may be summarized as follows: we have failed completely to effect the synthesis of amino-acids directly from glyoxals *in vitro* by acting on them or their acetals with ammonia or derivations of it. We have a considerable number of experiments under widely differing conditions with uniformly negative results, and it appears unnecessary to record these experiments in detail. On the other hand, we have obtained some evidence of the formation of optically active α -amino-acids from glyoxals *in vivo*, although, as will be shown later, it is doubtful if the change is a direct one. We have secured evidence indicating the synthesis *in vivo* of optically active leucine, α -amino-phenyl acetic acid and possibly of phenyl alanine from isobutyl, phenyl and benzyl glyoxals, respectively. We have also made experiments with glyoxal itself and with methyl glyoxal. In the case of glyoxal, which was used in the monomolecular form prepared by distilling ordinary glyoxal with phosphorus pentoxide and collecting the vapors in water, we obtained definite indications of the formation of glycine which was separated in the form of its β -naphthalene sulphonic acid derivative. The amount thus isolated was small but it appeared to be decidedly larger than the traces of glycine separated in blank experiments. That glycine was actually synthesized from glyoxal can hardly be affirmed with certainty. With methyl glyoxal we have been unable so far to demonstrate the formation of alanine, and this is the more surprising since much lactic acid is formed from the methyl glyoxal and from Embden's experiments the former substance would be expected to yield alanine.

In each case, the experiments were made by perfusing dogs' livers with defibrinated dogs' blood containing the glyoxal together with a small amount of ammonium bicarbonate. Special blank tests showed that provided the perfusion fluid contained relatively little saline, the yield of amino-acid obtained in experiments in which no glyoxal was added, was very small. But in all perfusions in which glyoxals were used, small amounts of other amino-acids, especially leucine, were invariably present. This fact added materially to the difficulty of isolating in a pure state any amino-acids formed synthetically from the glyoxals.

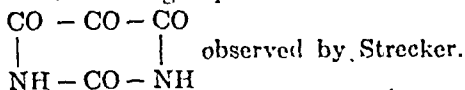
It must not be overlooked that these experiments are made under conditions far removed from normal. Under ordinary conditions, the concentration of glyoxals in tissues must be infinitely small and the addition of a relatively large amount of intensely reactive substance, such as glyoxals, to the blood used for perfusion of the liver, is undoubtedly objectionable. On the other hand, the presence of large amounts of glyoxalase in the blood undoubtedly converts much of the glyoxals into α -hydroxy-acids before reaching the liver. An additional difficulty is encountered in the fact that some glyoxals react with ammonia and amino-acids even in very dilute solution to give complicated cyclic compounds and are thus precluded from taking part in other reactions.³

For the isolation of the amino-acids from blood and liver, we made use of a method substantially similar to that employed by Embden and Schmitz.⁴ The amino-acids derived from the glyoxals were usually identified by conversion into uramido-acids by the action of potassium cyanate, followed by the extraction of the uramido-acids with ether. In some cases, for further identification, the uramido-acids were converted into hydantoins by heating with dilute hydrochloric acid.

The leucine obtained from isobutyl glyoxal was the natural laevo isomer and the phenyl alanine was also apparently the laevo variety, but the experiments on the formation of phenyl alanine from benzyl glyoxal were less satisfactory than the others, owing to the sparing solubility of benzyl glyoxal, rendering it impossible to use more than relatively small amounts for perfusion.

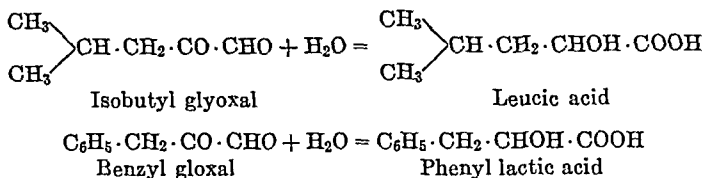
In addition to the amino-acids formed from the glyoxals on perfusion through the liver, we have obtained considerable

³ Reference may be made to an interesting reaction observed to occur between glyoxals and amino-acids. On warming their neutral aqueous solution together, a vigorous reaction occurs with the formation of a lower aldehyde, derived from the amino-acid. Alanine, for example, gives acetaldehyde, α -amino-phenylacetic acid gives benzaldehyde. The oxidation of an α -amino-acid to an aldehyde by means of a glyoxal containing the $-\text{CO}-\text{CO}-$ group recalls the similar oxidation of amino-acids by alloxan



⁴ *Biochem. Zeitschr.*, xxix, p. 423, 1910; xxxviii, p. 393, 1912.

amounts of the corresponding hydroxy-acids, glycollic, lactic, leucic, mandelic and phenyl lactic acids. The formation of glycollic, lactic and mandelic acids under these conditions has been previously recorded by us. These hydroxy-acids are undoubtedly formed from the action of glyoxalase upon the glyoxals, and in each case we have brought about the same result *in vitro* on digesting the glyoxal with an appropriate enzyme solution.



A curious fact presents itself in connection with the stereochemical relationships of these hydroxy- and amino-acids. Laevo-leucine is believed to be stereochemically related to laevo-leucic acid and gives the latter substance on treatment with nitrous acid. We also show in this paper that *l*-phenyl alanine gives *l*-phenyl lactic acid under similar conditions. No Walden inversion is believed to occur under the influence of nitrous acid in either of these reactions. Yet while on liver perfusion, isobutyl and benzyl glyoxals yield natural *l*-leucine and apparently *l*-phenyl alanine, we also obtain *d*-leucic acid and *d*-phenyl lactic acids. The significance, if any, attaching to these observations is not apparent, but it may be recalled that at present we have no precise evidence that leucic or phenyl lactic acids play any part in normal animal metabolism.

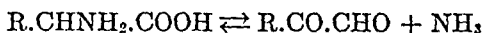
It may be noted that *d*-leucic acid has not been previously obtained. Its properties, other than sign of rotation, closely resemble those of the *l*-acid described by Scheibler and Wheeler.⁵ *d*-Phenyl lactic acid as obtained by us was identical in properties with the substance recently described by McKenzie and Wren⁶ which they prepared by resolving the inactive acid by means of morphine.

The foregoing demonstration of the synthesis *in vivo* of optically active amino-acids from glyoxals, raises the question of the

⁵ *Chem. Ber.*, xlv, p. 2684, 1911.

⁶ *Trans. Chem. Soc.*, xcvii, p. 1355, 1910.

mechanism of the reaction. As already stated in the introduction, we have shown that α -amino-acids under suitable conditions *in vitro* may yield glyoxals and the simplest conception of the relation between glyoxals and α -amino-acids, both *in vitro* and *in vivo*, would be to consider a direct chemical equilibrium between the two groups of substances:



It is very doubtful, however, if this simple scheme is actually representative of the facts concerning amino-acid synthesis. Certain observations which are admittedly incomplete appear to be opposed to the above representation. In the first place, we have been unable to observe as yet the synthesis of amino-acids from glyoxals outside the body, by direct addition of ammonia or derivatives of it. The usual action of ammonia upon glyoxals is to form cyclic iminazol derivatives, and while the latter substances are of biochemical interest, they are not known to be directly concerned with amino-acid formation. Liubawin⁷ has described the conversion of glyoxal into glycine by the successive action of ammonium cyanide and dilute sulphuric acid, but we have been unsuccessful both in repeating this experiment and in applying the reaction to higher glyoxals. We conclude, therefore, that a convincing demonstration of the direct formation *in vitro* of α -amino-acids from glyoxals is at present lacking.

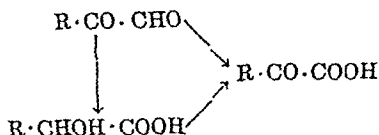
The possibility of glyoxals being converted into α -ketonic acids furnishes the basis for an alternative hypothesis to account for the formation of amino-acids from glyoxals in the liver, for it is well known from the work of Knoop⁸ and of Embden and Schmitz and others that α -ketonic acids may readily yield α -amino-acids both *in vivo* and *in vitro*.⁹ The possibility of α -ketonic acid formation from glyoxals is obvious and we have already demonstrated the formation of phenyl glyoxylic acid from phenyl glyoxal in the liver. The α -ketonic acids may be formed from glyoxals

⁷ Journ. Russian Chem. Soc., xiii, p. 329; xiv, p. 281, 1882.

⁸ Zeitschr. f. physiol. Chem., lxxi, p. 153, 1911.

⁹ An additional case of amino-acid synthesis in the liver from α -ketonic acids is described in the experimental part of this paper, namely, the formation of *D*- α -amino-phenyl acetic acid from the ammonium salt of phenyl glyoxylic acid.

either by direct oxidation or secondarily by the oxidation of α -hydroxy-acids formed from glyoxals by the action of glyoxalase.



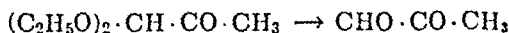
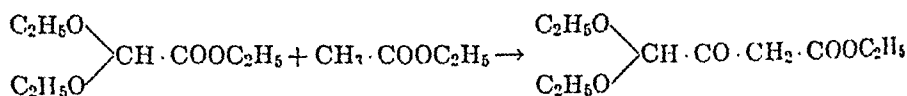
We were able to make an experiment on the fate of benzyl glyoxal in the animal organism which has some bearing on the above reactions. Benzyl glyoxal when fed to a rabbit gave rise to no detectable amount of phenaceturic acid in the urine, thus showing clearly that the first step in the decomposition of benzyl glyoxal, $\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CHO}$, does *not* consist in the removal of the aldehyde group by oxidation, with formation of a lower unsubstituted acid. If oxidation at the α -position had occurred phenyl acetic acid would have been formed and this, as is well known, is resistant to further oxidation in the body and would have been excreted in combination with glycine as phenaceturic acid.

An additional reason for considering the view that amino-acids when derived from glyoxals may originate by way of the α -ketonic acids is found in the impression that we have gained that the yield of amino-acid synthesized in the liver is at least as great or somewhat greater when ketonic acids are added to the perfusion fluid than when the corresponding glyoxals are employed. Much weight cannot be attached to this evidence for the reason already referred to, namely, that our experiments with glyoxals are carried out under conditions which are probably far less normal than when the neutral ammonium salt of a ketonic acid is employed. The glyoxals are of course intensely reactive substances and under normal conditions their concentration must be infinitely small, while in our experiments their concentration was relatively high. The presence of the $=\text{CO} \cdot \text{CHO}$ group characteristic of glyoxals would permit their union with other organic substances of the most diverse kind and it does not seem unreasonable to suppose that reactions effecting their decomposition *in vivo* are brought about while they are in combination with other substances. It is possible, for example, that amino-acid synthesis might take

place less readily from a free glyoxal than from a substance derived from the union of the aldehyde group of a glyoxal with some other organic compound. We hope to examine this question experimentally.

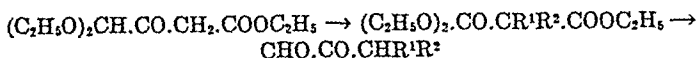
To sum up, it may be stated that optically active amino-acids may be formed from glyoxals *in vivo*, but it is not clear as to whether they are formed (a) directly by the addition of ammonia to free glyoxals or (b) formed from compounds derived by the union of glyoxals with other substances, or (c) formed secondarily from α -ketonic acids resulting from the oxidation of the glyoxals. Some evidence is adduced to show that the first type of change is perhaps the least probable, while in some cases amino-acids may be undoubtedly formed according to the last type of reaction.

Before passing to a description of the actual experiments, brief mention may be made of the synthesis of glyoxals. Both isobutyl and benzyl glyoxal have not been hitherto described. We have synthesized them by modifying the method which was employed by Wohl and Lange for the preparation of the acetal of methyl glyoxal. Isobutyl glyoxal is a yellowish green liquid, while benzyl glyoxal is a crystalline solid. Since the above experiments were concluded, we have devised a method for the synthesis of a variety of glyoxals which appears to be preferable to existing methods. We find that under suitable conditions diethoxyacetic ester condenses smoothly with acetic ester in the presence of sodium to give γ -diethoxyacetoacetic ester. This substance on treatment with potassium hydroxide is converted into the acetal of methyl glyoxal and on hydrolyzing the latter with dilute sulphuric acid, methyl glyoxal is obtained:



γ -Diethoxyacetoacetic ester is readily substituted with either one or two alkyl groups by the successive action of sodium ethylate and an alkyl halide. The alkylated esters may be converted

into the acetals of glyoxals by the action of alkali and thence into free glyoxals by dilute acid as in the case of methyl glyoxal:



By these reactions we have been able to prepare without difficulty methyl, ethyl, isopropyl, secondary butyl, isobutyl and phenylethyl glyoxals. A detailed account of these syntheses will be published shortly.

II. *Synthesis of isobutyl glyoxal.*

The general methods employed for the preparation of methyl glyoxal and its acetal by Wohl and Lange¹⁰ and by Meisenheimer¹¹ were applied to the synthesis of isobutyl glyoxal. Excess of magnesium-isobutyl bromide was allowed to react with the piperidide of diethoxyacetic acid, and by decomposing the resulting condensation product with strong ammonium chloride solution the acetal of isobutyl glyoxal was obtained, which on hydrolysis with dilute sulphuric acid yielded free isobutyl glyoxal. Although excess of the magnesium compound was used, the reaction between it and the piperidide was never complete, unchanged piperidide being always recovered on fractionating the acetal.

Acetal of isobutyl glyoxal. Ten grams (2.5 mols.) of magnesium ribbon and 70 cc. of dry ether were placed in a flask (500 cc.). A crystal of iodine was added followed slowly by a solution of 72 grams (3 mols.) of isobutyl bromide in 30 cc. of ether. The reaction starts promptly and is vigorous. By warming gently toward the end of the reaction all the magnesium may be dissolved.

The flask was then immersed in ice water and 36 grams (1 mol.) diethoxyacetpiperidide were dropped slowly into the solution. After about half the piperidide had been added, a grey, sticky solid separated out. When all the piperidide had been added, the flask was heated in a water bath at 50° for six hours and then allowed to stand over night. The ether was then poured off in order to remove unchanged piperidide as far as possible, fresh

¹⁰ *Chem. Ber.*, xli, p. 3612, 1908.

¹¹ *Ibid.*, xlv, p. 2635, 1912.

ether was added and the grey solid mass in the flask was decomposed by adding cold, strong ammonium chloride solution (150 grams in 500 cc. water) in small quantities at a time with constant shaking. When all the solid had gone into solution, the ether layer was tapped off and the aqueous solution was extracted three times with ether. The combined ether extracts were dried over sodium sulphate, the ether distilled off and the residual oil fractionated. Three fractions were collected:

- (1) 40-55° at 12-15 mm. with water-bath at 100°. Yield 5 grams.
- (2) 90-97° at 10-12 mm. with oil-bath at 130°. Yield 19 grams.
- (3) 90-120° at 1-2 mm. with oil-bath from 130°-180°. Yield 6.5 grams.

Fraction (2) contains the acetal in a fairly pure state.

Fractions (1) and (3) contain small quantities of the acetal together with unchanged isobutyl bromide and the piperidide of diethoxyacetic acid respectively.

Hydrolysis of isobutyl-glyoxal acetal. The acetal, a colorless highly refractive liquid with a pleasant sweetish smell, was hydrolyzed by boiling it with ten times its volume of 2 per cent (vol.) sulphuric acid for three hours under a reflux condenser. The solution at first becomes cloudy, but quickly clears again and the oil gradually assumes a light yellow-green color. After hydrolysis is complete, half the liquid is distilled off through a condenser and in this way an aqueous solution of isobutyl glyoxal, together with an oily layer of undissolved isobutyl glyoxal is obtained. This may be extracted with ether and it is interesting to note that on shaking out six times with ether, although the aqueous layer after the third extraction is quite colorless, the succeeding ether extractions are distinctly tinged with the typical yellow-green color of the free isobutyl glyoxal. The extraction of the glyoxal is incomplete even after repeated shaking with ether.

Isobutyl glyoxal is a yellow-green liquid which on distillation gives green vapors. It boils at 45°-46° at 12 mm. pressure. The pure substance resembles methyl glyoxal in smell but has in addition a rancid quality suggestive of valeric acid. It was characterized by conversion into the crystalline semicarbazone and dinitrophenylhydrazone.

Semicarbazone of isobutyl glyoxal. This substance was prepared by warming a solution of the glyoxal dissolved in a little

alcohol with an aqueous solution of semicarbazide hydrochloride. A separation of white crystals of the mono-semicarbazone occurs almost immediately. The substance is sparingly soluble even in hot alcohol, but dissolves somewhat more readily in boiling water, crystallizing on cooling in colorless transparent prisms melting sharply at 249–250°.

ANALYSIS: 0.0569 gram gave 0.0140 gram N = 24.7 per cent N.
 $C_{17}H_{13}O_2N_3$ requires 24.6 per cent N.

Isobutyl-glyoxal dinitrophenylhydrazone. This substance was prepared by adding excess of *p*-nitrophenylhydrazine dissolved in dilute sulphuric acid to a solution of the glyoxal. The hydrazone is at once precipitated in quantitative yield. The substance is sparingly soluble in alcohol and toluene but may be conveniently crystallized by dissolving it in boiling nitrobenzene and then adding toluene. It crystallizes in scarlet needles melting at 288–290° with evolution of gas.

ANALYSIS: 0.0901 gram gave 0.0197 gram N = 21.9 per cent N.
 $C_{13}H_{20}O_4N_6$ requires 21.9 per cent N.

III. The formation of l-leucine and d-leucic acid from isobutyl glyoxal in the liver.

The following description is typical of three separate experiments which furnished identical results. The liver (339 grams) of a dog weighing 14 kgm. was perfused as previously described, with 1420 cc. of undiluted defibrinated dogs' blood. Small quantities of separate mixtures of 5 grams of isobutyl glyoxal in 50 cc. of water and of 3 grams of ammonium bicarbonate in 150 cc. of water were alternately added to the perfusion blood. After about half an hour the whole of both solutions had been added and the perfusion was continued for an additional hour. The liver was then washed by perfusing salt solution (200 cc.) through it and was then rapidly minced and at once extracted with boiling water. The aqueous extract together with the blood and washings were then precipitated with mercuric chloride in hydrochloric acid solution according to Schenk's directions. The filtrate was freed from mercury with hydrogen sulphide and the filtrate, after careful neutralization with sodium hydroxide, was evaporated to

small bulk *in vacuo*. The liquid was then acidified with phosphoric acid (3 cc.) and leucic acid removed by extraction with ether.

On evaporation of the ether, a crystalline residue of crude leucic acid, contaminated with a little lactic acid, was obtained. The acidity and optical rotation corresponded to about 1.8 grams of the acid. The yield in other similar experiments amounted to as much as 2 grams. The *d*-leucic acid was purified by crystallization from a mixture of ether and petroleum and crystallized in prisms melting at 80–81° after slight softening at 78°. The optical rotation was observed in normal sodium hydroxide solution.

$$c = 1.16; l = 2; \alpha = + 0.66^\circ$$

$$[\alpha]_D = + 27.6$$

Scheibler and Wheeler (*loc. cit.*) give the melting point of *l*-leucic acid as 81–82° with sintering at 78° and the specific rotation in normal sodium hydroxide as -27.8° . *d*-Leucic acid has not been previously obtained in a pure state.

The remainder of the *d*-leucic acid was dissolved in water and added to a solution of zinc acetate. The precipitated zinc leucate was recrystallized from very dilute alcohol. The air-dried salt was analyzed:

0.0664 gram lost 0.0034 gram H_2O at 130° and gave on ignition 0.0157 gram zinc oxide.

	Calculated for ($C_6H_{11}O_7$): $Zn.H_2O$:	Found:
Zn.....	18.9 per cent	19.0 per cent
H_2O	5.2 per cent	5.1 per cent

The aqueous solution from which the leucic acid had been removed by ether was neutralized with potassium hydroxide and then concentrated on the water bath with the addition of 2 grams of potassium cyanate. Subsequently the evaporation was repeated after the addition of more water and cyanate. By this procedure the leucine present in the solution was converted into α -uramido-isobutylacetic acid. The alkaline solution was then strongly acidified with phosphoric acid and extracted with ether for twelve hours in a continuous extractor. After the extraction had been continued for a very short time, a crystalline deposit of α -uramido-isobutylacetic acid, the uramido-acid derived from

leucine, appeared in the ether flask and steadily increased in quantity. At the end of the extraction the ether was concentrated and poured off from the crystals. On complete evaporation of the ether and subsequent treatment with water a small somewhat less pure second crop of crystals was obtained. The yield of crystalline uramido-acid in different experiments varied from 0.11 to 0.3 gram. Since the uramido-acid has an extremely low optical rotation, it was converted into the more characteristic hydantoin by boiling with dilute hydrochloric acid. After repeated crystallization from water, the isobutyl hydantoin melted at 218–219°. The specific rotation of three separate preparations observed in 0.5 per cent solution in 50 per cent alcohol varied from -70 to -95° . The variations in rotation are probably due to partial racemization of the hydantoin during the treatment with hydrochloric acid, for we have observed a similar change with benzyl hydantoin.¹²

The *l*-hydantoin was analyzed as follows:

0.0986 gram gave 0.0178 gram N = 18.0 per cent N.

$C_7H_{12}N_2O_2$ requires 18.0 per cent N.

The hydantoin was identical in its properties with specimens of the substance obtained by other methods.

IV. *The action of glyoxalase upon isobutyl glyoxal.*

A solution containing active glyoxalase was prepared by extracting fresh minced dog's liver with ten parts of water. Fifty cc. of the extract were shaken with 0.75 gram of freshly prepared isobutyl glyoxal and excess of precipitated chalk. The mixture was incubated for eighteen hours and then heated in the water bath for two minutes after adding 20 grams of ammonium sulphate. After cooling, excess of phosphoric acid was added and the mixture filtered from precipitated protein. On extracting the filtrate with ether and evaporating the solvent a crystalline residue of *d*-leucic acid (0.25 gram) was obtained. The acid was purified by dissolving in ether and precipitating with petroleum ether. The acid melted at 80–81° after softening at 78°, and had a specific rotation in normal sodium hydroxide of $+27.6^\circ$. It also gave the characteristic sparingly soluble zinc salt and was identical with

¹² This *Journal*, xvii, p. 29, 1914.

the product obtained on liver perfusion, as described in the preceding section. It would appear from the rotation and melting point that the dextro-acid was formed exclusively.

V. *Synthesis of benzyl glyoxal.*

This substance was synthesized by similar methods to those used in the preceding preparation. Benzyl magnesium chloride was allowed to act on the piperidide of diethoxyacetic acid, the product decomposed with strong ammonium chloride solution and the resulting acetal hydrolyzed with dilute sulphuric acid. The isolation of pure benzyl glyoxal was rendered difficult by the fact that much dibenzyl was formed during the process, but eventually, by taking advantage of the relative insolubility of benzyl glyoxal in petroleum ether, this substance was isolated in the pure state.

Acetal of benzyl glyoxal. Five grams (2.5 mols.) of magnesium ribbon and a solution of 31 grams (3 mols.) of benzyl chloride in 50 cc. of dry ether were mixed in a 200-cc. flask. The reaction was started by adding a crystal of iodine and gently warming. After the first brisk action subsided, the flask was warmed on the water bath for about two hours. The solution of benzyl magnesium chloride was then poured off from undissolved magnesium into a 500-cc. flask, cooled in ice and 18 grams (1 mol.) of diethoxyacetic piperidide were dropped slowly into the solution. A grey, solid mass was formed and the flask was heated at 50° in a water bath for six hours. The ether layer was then poured off and in this way a considerable quantity, though by no means all, of the dibenzyl was removed. Fresh ether was added and the solid mass in the flask was then decomposed with strong ammonium chloride solution. The ether layer was separated and the liquid extracted three times with ether. The ether extract was dried over sodium sulphate and after removal of the ether, the yellow oil remaining was distilled *in vacuo*. A separation of dibenzyl from the acetal of benzyl glyoxal was found to be impossible by fractional distillation. A yield of 21 grams of a pale yellow oil, consisting mainly of a mixture of dibenzyl and benzyl-glyoxal acetal, was obtained which boiled at 90°–125° at 1–2 mm. pressure, the oil bath being heated up to 180°.

It is possible to crystallize out of this mixture a fair quantity of dibenzyl by adding a little alcohol and seeding with a crystal of dibenzyl. It was found better, however, not to attempt separation at this point, but to hydrolyze the mixture with ten times its volume of 2 per cent (vol.) sulphuric acid, boiling under a reflux condenser for three hours.

Benzyl glyoxal. The hydrolysis mixture, after three hours' boiling, was distilled through a condenser. Crystals, which were collected, formed in the condenser, and the aqueous distillate was returned to the flask and redistilled. This was repeated until no more formation of crystals in the condenser was observed. The crystals (8.3 grams from 21 grams of the acetal fraction), consisting of a mixture of dibenzyl and benzyl glyoxal, were pressed out on porous plate and dried in the air. They were then warmed with a small quantity of petroleum ether, the solution was allowed to cool and the insoluble residue, consisting of practically pure benzyl glyoxal, was filtered off. About 3 grams of benzyl glyoxal were obtained from each preparation.

Benzyl glyoxal, thus prepared, crystallizes from toluene or petroleum ether in the form of long, white, glistening needles, softening at about 115° and melting at 120° – 122° . It can also be recrystallized from 50 per cent alcohol. Although a pure white substance, its solutions in any of these solvents on warming rapidly assume a yellowish tint.

In aqueous solution it gives a canary-yellow precipitate with benzhidine, and this compound dissolves in concentrated sulphuric acid with slight green fluorescence.

Analysis of benzyl glyoxal crystallized from toluene:

0.1583 gram gave 0.4234 gram CO_2 and 0.0751 gram H_2O .

	Found:	Calculated for $\text{C}_7\text{H}_8\text{O}_2$:
C.....	72.94	72.97 per cent.
H.....	5.27	5.4 per cent.

Benzyl-glyoxal dinitrophenylhydrazone. This compound was prepared by adding a solution of *p*-nitrophenylhydrazine in dilute sulphuric acid to a hot aqueous solution of benzyl glyoxal. A precipitate, yellow at first, but rapidly becoming scarlet on boiling, was formed. This was filtered off, washed with warm 50 per cent alcohol and dried for one hour at 110° . It can be crystallized from

a mixture of nitrobenzene and toluene and separates out in scarlet needles, which melt at 275–278°.

ANALYSIS: 0.1005 gram gave 0.0202 gram N = 20.1 per cent N.

$C_{21}H_{18}N_6O_4$ requires 20.1 per cent N.

Benzyl-glyoxal dinitrophenylhydrazone is very difficultly soluble in cold sodium hydroxide, but on warming it goes slowly into solution giving a pure blue color. Addition of a small quantity of alcohol causes it to dissolve much more rapidly with the production of an intense blue solution.

VI. Liver perfusion experiments with benzyl glyoxal.

Our perfusion experiments with benzyl glyoxal were somewhat unsatisfactory, owing to the difficulty of obtaining a sufficient quantity of the glyoxal dissolved in the perfusion fluid. Pure benzyl glyoxal requires about a thousand parts of water for its solution at room temperature and the amount that we could dissolve in salt solution at blood temperature was necessarily small. Furthermore, the free dilution of the perfusion blood with salt solution appears to lead to greater autolysis of liver tissue than otherwise occurs. Various devices to circumvent the difficulty of solubility were tried, with indifferent success. Two experiments were made in which as much benzyl glyoxal as possible was dissolved in 300–450 cc. of warm saline and then mixed with a large volume of dogs' blood, but it is doubtful if more than a gram of the glyoxal was actually retained in solution. The smell of benzyl glyoxal which was marked at the commencement of the perfusion completely disappeared at the close and no unchanged substance could be detected. The acetoacetic acid formation was slight, amounting to only 50.1–58.5 mgm. This result is in harmony with the recent results of Embden and Baldes¹³ in which it is shown that closely related phenylpyruvic acid not only yields no acetoacetic acid but inhibits its formation from other substances.

The liver and blood were treated precisely as described in Section III on the perfusion of isobutyl glyoxal. The first ether extract gave in each case a solid residue of crude *d*-phenyl lactic acid (0.25–0.4 gram). On recrystallization from water, the acid

¹³ *Biochem. Zeitschr.*, lv, p. 301, 1913.

was readily purified and crystallized in long glassy needles melting at 124–126°. The analysis gave results in satisfactory agreement with those of phenyl lactic acid. The specific rotation was determined in aqueous solution:

$$c = 0.857; l = 2.0; \alpha = +0.48^\circ; \\ [\alpha]_D = +24.8^\circ$$

McKenzie and Clough give 124–125° for the melting point and +22.8 for the specific rotation of an aqueous solution containing 2.56 per cent of the acid.

The uramido-acid fraction (0.1–0.2 gram) obtained from the benzyl glyoxal perfusions was a white crystalline solid, sparingly soluble in ether resembling *d*- β -phenyl- α -uramidopropionic acid. Its melting point, like the latter substance, was about 193–194° but was not sharp. Pure *d*- β -phenyl- α -uramidopropionic acid from *l*-phenylalanine melts at 195–196°. ¹⁴ On boiling with dilute hydrochloric acid, it gave a good crystalline hydantoin which was strongly laevorotatory in alcoholic solution. On repeated crystallization, it was found impossible to isolate pure benzyl hydantoin as the substance was contaminated with some other substance of higher melting point. On analysis the crude hydantoin was found to contain 15.7 per cent of nitrogen compared with 14.7 for pure benzyl hydantoin. The melting point of the uramido-acid and the laevorotation of the hydantoin furnish evidence in favor of the assumption of the formation of *l*-phenyl alanine from benzyl glyoxal, but cannot be regarded as finally conclusive.

VII. *The action of glyoxalase upon benzyl glyoxal.*

These experiments were made in precisely the same fashion as those recorded under Section IV. The crude phenyl lactic acid obtained on evaporation of the ether was purified by dissolving in sodium carbonate solution and shaking with ether. On subsequently acidifying and again extracting with ether, the acid was readily obtained as a white solid, crystallizing from water in long needles melting at 124–126°. The melting point and specific rotation of the substance in aqueous solution (+24.8°) showed that *d*-phenyl lactic acid had been formed practically exclusively, as was also the case with the experiments in Section VI.

¹⁴ This *Journal*, xvii, p. 34, 1914.

VIII. Fate of benzyl glyoxal in the animal body.

The sparing solubility of benzyl glyoxal made satisfactory experiments on its fate in the body somewhat difficult. At first we hoped to give intravenously to dogs considerable doses of the glyoxal dissolved in weak alcohol. The amount of solvent necessary made this method of little use. Finally we chose to give the glyoxal (0.75 gram) dissolved in alcohol (5 cc.) by stomach tube to rabbits. After the alcohol solution had been washed in about 50 cc. of water was given. The urine was collected for the next twenty-four hours. No toxic symptoms were observed and no unchanged glyoxal appeared in the urine. The urine was acidified with phosphoric acid and extracted with ether in a continuous extractor. The ether residue was treated with hot water and filtered. The solution showed a feeble dextrorotation probably due to a trace of *d*-phenyl lactic acid, but the amount was trifling. On concentrating the aqueous solution, a small quantity of hippuric acid (0.09 gram) was obtained, but no other substance could be detected. Phenaceturic acid was absent. The amount of hippuric acid was no more than would be normally present in the urine.

IX. The conversion of l-phenylalanine into l-phenyl lactic acid.

For the purposes of the following experiments, we made use of a phenylalanine preparation which Dr. T. B. Osborne kindly gave us. The amino-acid had a specific rotation in 1 per cent aqueous solution of -18.3° corresponding to a mixture of slightly more than half of the active acid with the inactive variety.

l-Phenylalanine hydrochloride (0.5 gram) was dissolved in dilute sulphuric acid (7.6 cc.) and while cooled to 0° sodium nitrite (0.3 gram) dissolved in 2 cc. of water was slowly added through a fine capillary tube. The solution was allowed to stand in ice for three hours and then at room temperature over night. The solution was then extracted with ether. On evaporation of the solvent a crystalline residue of phenyl lactic acid (0.3 gram) was obtained. The acid was separated from traces of oily impurity by dissolving in water. The optical activity was determined in aqueous solution:

$$c = 1.56; l = 2.0; \alpha = -0.17^\circ.$$
$$[\alpha]_D = -13.3^\circ$$

This rotation corresponds to somewhat more than 50 per cent of the laevo acid, and since it was prepared from *l*-phenylalanine of about the same optical purity, it appears that no significant racemization had occurred during the reaction.

X. Formation of d- α -amino-phenylacetic acid from phenyl glyoxal and phenyl glyoxylic acid in the liver.

Four separate perfusions with phenyl glyoxal hydrate (5 grams) or phenyl glyoxylic acid (5 grams) were carried out. In the case of phenyl glyoxal, ammonium bicarbonate (2 grams) was added gradually to the perfusion fluid while the phenyl glyoxylic acid was neutralized with ammonia before the commencement of the experiment. The blood and liver were analyzed precisely as in the case of the isobutyl glyoxal perfusion. The first ether extract from the phenyl glyoxal perfusions gave about 2 grams of *l*-mandelic acid and a little phenyl glyoxylic acid, while the phenyl glyoxylic acid perfusion gave relatively little *l*-mandelic acid (0.1 gram) but much unchanged substance together with benzoic acid. We shall not refer further to these substances since their formation has been described previously, either by Neubauer¹⁵ or ourselves.

The *d*-amino-phenylacetic acid was identified by conversion into the uramido-acid in the usual way. The yield of uramido-acid in each experiment was about 0.25 gram. The substance obtained from phenyl glyoxylic acid melted after one crystallization from water at 204–205° with effervescence and after two further crystallizations at 211°. The substance was strongly dextrorotatory but the specific rotation of the once crystallized material observed in 1 per cent solution in normal ammonia (+ 67.5°) was much below the full value for optically pure material (see later), but served to indicate definitely the presence of the dextro acid. Indications were obtained of the presence of some uramido-acid derived from leucine. The twice crystallized substance on analysis gave 14.7 per cent of nitrogen compared with a theoretical value of 14.43 per cent.

¹⁵ *Deutsch. Arch. f. klin. Med.*, xcv, p. 232, 1909.

The uramido-acids obtained from the phenyl glyoxal perfusions resembled the foregoing substance closely. On repeated crystallization, the substance melted with effervescence at 210° and gave on analysis 14.7 per cent of nitrogen. On boiling with dilute hydrochloric acid it gave a hydantoin melting at 182° . The fact that the hydantoin showed a distinct laevorotation was evidence that the original uramido-acid was not strictly pure, for phenyl hydantoin prepared in the usual way from active α -uramido-phenyl acetic acids is optically inactive. As stated before, we have in almost all cases observed the presence of the uramido-acid derived from leucine in the glyoxal perfusions and the amount of product derived from the glyoxal is insufficient for a thoroughly complete purification.

A comparison of the data given above with those recorded below for pure *d*- α -amino-phenyl acetic acid and phenyl hydantoin and the corresponding data for the leucine derivatives can hardly leave the question of the formation of *d*- α -amino-phenyl acetic acid from phenyl glyoxal and phenyl glyoxylic acid in doubt.

XI. Preparation of α -uramido-phenylacetic acids and phenyl hydantoin.

Laevo- α -uramido-phenylacetic acid was prepared for purpose of comparison, from *l*- α -amino-phenyl acetic acid. The latter acid was obtained from the urine of a small dog which received 9 grams of the inactive acid in the course of two days. As shown by Neubauer, the laevo component is largely excreted while the dextro compound is oxidized. The urine was acidified with acetic acid so as to restrain uramido-acid formation through interaction with urea, filtered hot and then concentrated *in vacuo*. Almost 3 grams of the amino-acid were obtained by direct crystallization. For purification it was dissolved in hot dilute ammonia, boiled with charcoal and then crystallized from the concentrated solution. The *l*- α -amino-phenyl acetic acid in 1 per cent solution in normal hydrochloric acid had a specific rotation of -154° . The pure acid under very slightly different conditions has a rotation of -157.8° according to E. Fischer.¹⁶

¹⁶ *Chem. Ber.*, xli, p. 1290, 1908.

The active α -uramido-phenyl acetic acid was prepared in the usual way by evaporation of a solution of equal weights of the *l*-amino-acid and potassium cyanate. On acidification and recrystallization from water, the uramido-acid was obtained pure and in excellent yield. The melting point of the substance varies considerably according to the rate of heating. If the heating be slow, as low a melting point as 203° may be observed, while with rapid heating the melting point is constant at $209-211^{\circ}$. Neubauer observed a melting point of 208° for the substance he obtained from urine but the optical rotation of his substance was considerably lower than ours. The rotation was conveniently observed in 1 per cent solution in normal ammonia.

$$c = 1.0; l = 2 \text{ dm.}; \alpha = -2.67^{\circ}.$$
$$[\alpha]_D = -133.5^{\circ}.$$

The rotation was not materially changed on recrystallization, although it is probable that the observed rotation is slightly low. The rotation observed in 50 per cent alcohol is considerably higher.

On boiling the α -uramido-acid with twenty parts of 5 per cent hydrochloric acid, it is converted with complete racemization into phenyl hydantoin melting at $184-185^{\circ}$. This racemization is analogous to but more complete than the racemization recently observed by us in the formation of benzyl hydantoin from active β -phenyl- α -uramido-propionic acid.

For purposes of comparison, we have prepared inactive α -uramido-phenyl acetic acid and phenyl hydantoin starting from inactive α -amino-phenyl acetic acid. We find the melting points of these substances to be materially higher than those recorded by Pinner,¹⁷ who ascribed a melting point of 178° to both substances. We find that inactive α -uramido-phenyl acetic acid crystallizes from water in fine prisms melting with effervescence at $196-196.5^{\circ}$. Inactive phenyl hydantoin crystallizes from water in plates and prisms and melts at $184-185^{\circ}$ without decomposition.

¹⁷ *Chem. Ber.*, xxi, p. 2320, 1888.

XII. Liver perfusion experiments with glyoxal and methyl glyoxal.

The glyoxal for these experiments was used in the monomolecular form prepared by distilling ordinary glyoxal with phosphorus pentoxide according to the method of Harries and Temme.¹⁸ The glyoxal vapors were passed through three flasks each containing 50 cc. of water cooled with ice. Three separate distillations using 5 grams of ordinary glyoxal served to give a solution containing about 3 grams of the active product. Sodium chloride in suitable amount was added to the aqueous solution and it was filtered after warming to 37°. The glyoxal solution was added gradually to the blood used for perfusion and a solution containing an equivalent amount of ammonium bicarbonate was also added by degrees. After perfusion for ninety minutes the blood and liver were treated with Schenck's reagent and the mercury removed with sulphuretted hydrogen in the usual way. The neutralized filtrate was concentrated, then acidified with dilute sulphuric acid and precipitated with phosphotungstic acid. The filtrate was freed from phosphotungstic acid, sulphuric acid and ammonia by means of barium hydroxide and the concentrated filtrate acidified with phosphoric acid and extracted with ether in a continuous extraction apparatus for twenty-four hours. In each case a large yield of glycollic acid was obtained which was identified as previously described by us by conversion into the calcium salt and then back into the free acid.

The aqueous solution remaining after extraction with ether was then examined for glycine by means of the β -naphthalene sulphonic chloride method as described by Bingel.¹⁹ It is unnecessary to reproduce the details of this method but it may be mentioned that the reaction with the sulphonic chloride was conveniently carried out at 25° in a "Thermos" bottle. We have obtained from the best of our glyoxal perfusions slightly over 0.1 gram of recrystallized β -naphthalene-sulphoglycine melting at 154–155°. The purification of the substance involves great loss so that the actual amount present must have been larger. The melting point was unchanged on mixing with the pure compound prepared directly from glycine. In blank perfusions we have in some cases been

¹⁸ *Chem. Ber.*, xl, p. 165, 1907.

¹⁹ *Zeitschr. f. physiol. Chem.*, lvii, p. 382, 1908.

able to detect the presence of glycine in the blood and liver by following the above method, but the yield of substance was never more than a few milligrams. It seems possible, therefore, that glycine may have been formed from glyoxal, but it cannot be regarded as definitely proved.

For the methyl glyoxal perfusions we used in each case 5 grams of the substance freshly prepared by hydrolyzing methyl glyoxal acetal with decinormal sulphuric acid and removing the latter by means of barium hydroxide. An equivalent amount of ammonium bicarbonate was added slowly to the perfusion blood. The blood and liver were examined for alanine precisely as described by Embden and Schmitz, but in no case were we able to definitely satisfy ourselves of the presence of *d*-alanine. We were no more successful when we applied a method based on the extraction by ether of the uramido-acid, derived from *d*-alanine and its subsequent conversion into the very soluble *l*-methyl hydantoin. In each case we were able to detect the presence of traces of leucine, but failed to identify *d*-alanine. At present, therefore, we are without any evidence pointing to a direct synthesis of *d*-alanine from methyl glyoxal.

UREA: ITS DISTRIBUTION IN AND ELIMINATION FROM THE BODY.

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I. INTRODUCTION.

Studies upon urea have been prosecuted unremittingly since its discovery in urine in 1773 by Rouelle.¹ The interest of this substance to the physiologist and to the clinician has been from two standpoints, namely that of protein metabolism, and that of renal function. The experiments recorded in this article bear directly, it is felt, upon both of these subjects.

Ever since Prevost and Dumas² in 1821, isolated urea from the blood after extirpation of the kidneys, many investigations have been made upon the urea content of the blood in health and disease. Naturally these studies have been extended to the analysis of various animal fluids and tissues, qualitatively and quanti-

¹ *Journ. de med.*, Nov. 1773; quoted by Chattaway: *Chemical News*, xcix, p. 121, 1909.

tatively, for urea. The qualitative part of the problem has been solved much more satisfactorily than the quantitative. This has been due mainly to the existence of more certain and exact methods for the recognition of urea than for its estimation.² The specific method recently suggested by one of us⁴ for urea determinations lends itself readily to a quantitative study of urea in the animal organism. The older methods have been at fault in two respects; namely a loss of urea by the procedures used in its isolation, and the simultaneous determination of other substances, along with the urea. This error is of much greater significance in dealing with tissues than with urine and blood, apparently on account of the occurrence of interfering substances in much larger amounts.

The occurrence of urea in animal organs has been proven conclusively by its isolation in analytically pure condition.⁵ Such isolation in a state of purity is necessarily attended with great loss, and tells little about the actual content of the tissues in urea. We feel that the numerous investigations by various quantitative urea methods at one time and another are not satisfactory, owing to the inaccuracies attending these methods. This is well shown by the contradicting and confusing statements occurring in the literature. Picard⁶ found the muscles, brain, and liver of a cat to contain very different amounts, the content of muscle being about five times as great as that of the liver per unit of weight.

Gscheidlen⁷ found in the different organs of a dog the urea content to be about the same as the blood (0.02 per cent) with a high content in the spleen and none in muscle. Kaufmann⁸ states that the organs contain less urea than the blood, and that, of the organs, the liver contains the most. Schöndorff,⁹ in his researches on urea in the animal organism, found in a well-fed dog that the

² *Ann. de chim. et de. phys.*, 2me serie, xxiii, p. 90, 1823.

³ For a good collection of the literature on urea in blood, body fluids and tissues see Schöndorff: *Pflüger's Archiv*, lxxiv, p. 307, 1899.

⁴ Marshall: this *Journal*, xiv, p. 283, 1913; xv, p. 487, 1913.

⁵ For literature see Schöndorff; *Pflüger's Archiv*, lxxiv, p. 330, 1899.

⁶ *Compt. rend. de l'Acad. des Sci.*, lxxxvii, pp. 533 and 993, 1878.

⁷ Gscheidlen: *Studien über den Ursprung des Harnstoffs in Thierkörper*. Leipzig, 1871.

⁸ *Arch. de physiol.*, v, serie 6, p. 543.

⁹ *Loc. cit.*

liver, spleen, pancreas and brain, contained about the same amount of urea as the blood (0.1115–0.1228 per cent) while the heart contained slightly more and the muscles slightly less. The values, however, are too high even for blood by his method, and would probably be still more erroneous for tissues. A great many other investigations have been made on single organs or tissues, which differ very widely in the figures obtained. The opinion that the liver contains more urea than any other organ has gained much credence, and is not infrequently repeated today as an argument for the liver as the seat of urea formation.¹⁰ Herter's¹¹ work rather confirmed this idea. He found the urea content of the liver more than twice as great as that of muscle and brain. However, he realized the inaccuracies of the methods employed, the measurement of the nitrogen evolved by sodium hypobromite, and placed no great dependence on the absolute results.

Certain indirect evidence has accumulated which shows that the tissues must act as a store-house for urea. The increases in the urea content of the blood in certain nephritics along with the small amounts in the urine fail to account for the total urea of the metabolism. This fact is better illustrated by the rapid disappearance of urea injected into the blood stream, without a corresponding increased elimination by the kidneys.¹²

In relation to this absorption of urea by the tissues we would especially call attention to the work of Grehant and Quinquaud, who showed directly an increase in the urea content of tissues after injections of urea, and to the recent work of Folin and Denis¹³ on protein metabolism, using new and more accurate methods for the determination of non-protein nitrogen and urea. In studying absorption from the ligated intestine of cats, they found that after injecting 100 cc. of a 4 per cent urea solution into the ligated loop, the urea was readily absorbed and transported to the muscles. The muscular tissue, however, was the only one analyzed.

¹⁰ Schäfer's *Text book of Physiology*, Vol. I, p. 902.

¹¹ Herter and Wakeman: *Journ. of Exp. Med.*, iv, p. 117, 1899; Herter: *Johns Hopkins Hospital Reports*, ix, p. 69, 1900.

¹² Grehant and Quinquaud; *Journ. de l'anat. et physiol.*, xx, p. 393, 1884; Herter: *loc. cit.*, and also von Noorden: *Metabolism and Practical Medicine*, Vol. II, p. 488.

¹³ This *Journal*, xi, p. 87, 1912; xii, p. 141, 1912.

In view of our findings, a comparison of the behavior of urea with that of other substances normally occurring in the blood and tissues is of interest. The distribution of chlorides has been studied by Nencki and Sumanowski¹⁴ in normal dogs, and more recently by Wahlgren¹⁵ in normal animals and those in which an increase had been produced by intravenous injections of salt. Great variations in the percentage content of the organs exist, the greatest concentrations occurring in the skin, blood and lungs, with less than one-third the concentration of the blood present in muscle. When added to the organism in excess this substance finds its way to the organs in different percentages, and its movements are independent of the simultaneous movements of water in the organism. Carbohydrate, in the form of glycogen, is known to occur in largest concentration in the liver and muscles.

Sassa¹⁶ finds β -oxybutyric acid to be distributed in normal animals uniformly in all the organs, although when excessive in amount it is more concentrated in the liver. Van Slyke and Meyer¹⁷ in their studies on the fate of amino-acids injected into dogs, have made numerous determinations of the amino-nitrogen content of tissues. They found the amino-acids of the blood to maintain an equilibrium with those of the tissues, although the tissues contain 5-10 times as much. The distribution was not equal for all tissues, and in the case of muscles a definite upper limit exists, above which it is impossible to raise the amino-acid content.

Turning to studies actuated by interest in renal function, it is seen that workers early hypothecated a retention of urea in nephritis, and thought of its possible toxicity. Vauquelin and Segalas¹⁸ who first injected urea into the blood, used small quantities and did not interfere with its excretion. Therefore, they noted no toxic effect. The same is true of many who followed them. Hammond¹⁹ interfered with the kidneys, and then found injections of urea fatal. Grehant and Quinquaud²⁰ determined lethal doses of urea. They state that a concentration of 0.6 gram per 100 cc. of blood is fatal to dogs.

Herter and Wakeman²¹ in 1899 performed a series of infusions of urea, with and without nephrectomy or ligation of the ure-

¹⁴ *Arch. f. exp. Path. u. Pharm.*, xxxiv, p. 313, 1894.

¹⁵ *Ibid.*, lxi, p. 97, 1909.

¹⁶ *Biochem. Zeitschr.*, lix, p. 362, 1914.

¹⁷ *This Journal*, xvi, p. 197, 1913.

¹⁸ *Journ. de physiol. exp. et pathol. par F. Magendie*, ii, No. 1, p. 331, 1822.

¹⁹ *No. Amer. Medico-chir. Rev.*, ii, no. 2, p. 287, 1858.

²⁰ *Journ. de l'anat. et physiol.*, xx, p. 393, 1884.

²¹ *Journ. of Exp. Med.*, iv, p. 117, 1899.

ters. By using concentrated solutions they were able to cause the death of intact dogs. The fatal termination usually occurred after about 1 per cent of the body-weight of urea had been introduced, but if the infusions were performed slowly the kidneys would rapidly eliminate a good proportion of the urea. They concluded that a dog's kidney can excrete, at its maximum capacity, 1.5 grams urea per gram kidney in twenty-four hours. The maximum urinary concentration of urea obtained by them was over 30 grams per liter, and the maximum excretion 105 cc. of urine in one hour (dog weighing 19 lbs.).

To recapitulate, it may be said that estimations of the urea content of organs, with conclusions drawn from them, are conflicting and unsatisfactory; that retention of urea is a regular occurrence in certain cases of severe nephritis; that the amount of urea in the blood does not account for the amount theoretically present in the organism; that there is evidence of an increase also in the urea content of tissues in cases where the blood urea is abnormally high; and that urea may exert a toxic action. The extent to which urea may be stored in the tissues has never been accurately determined.

The present investigation was undertaken to determine the urea content of the various tissues of the animal body, and the relation of this percentage to that of the blood. The study of the distribution when abnormally large amounts of urea are present in the organism has been approached by three means: 1. Analysis of tissues before and after the injection of large amounts of urea; 2. Analyses of tissues of animals whose kidney function had been interfered with, with or without urea injections, and 3. Analyses of human tissues obtained from autopsies of nephritics. Apparently no limit exists for the tissues in absorbing urea, for up to lethal doses it is taken up readily. The rapidity with which this equilibrium between the blood and tissues is established, has also been determined, as well as the rate at which the organism frees itself of abnormally large amounts of urea when injected. Certain observations confirmatory of Herter's work on the symptoms indicating the toxicity of large amounts of urea are also included.

II. EXPERIMENTAL.

1. *Methods of analysis.*

The urea determinations in urine and blood were carried out by the use of urease according to the methods described by one of us.²² In the cases where whole blood was used instead of serum, 5-cc. portions were drawn with a pipette and needle as described by Folin and Denis,²³ transferred to 2 cc. of 1 per cent sodium oxalate, treated with urease, and ammonia determined by the usual technique. Unless the determination was completed in a few hours, a second 5-cc. portion of blood was run as a control without the addition of enzyme. The urea content of bile, cerebrospinal fluid and the various organs was estimated by the method recently described by one of us. For bile and cerebrospinal fluid the technique was exactly the same as used for blood, while tissues were extracted with alcohol, as described in the former publication.²⁴

The method as applied to the estimation of the urea content of the various organs should be described in more detail.

The organs were removed immediately after death and portions, usually 10 grams each, weighed carefully. These were transferred to 95 per cent alcohol, allowed to remain over night, ground thoroughly with sand, and returned to the same alcohol, about 100 cc. of alcohol being used.

After standing for twenty-four hours or more the mixture was filtered with suction and thoroughly washed with warm 95 per cent alcohol. In experiments in which a large percentage of urea was expected in the organ, the residue was reground, once or twice with alcohol. This procedure effects a quantitative extraction of the urea. The alcoholic filtrate²⁵ was evaporated at a low temperature on the water bath to about 5 cc. and diluted with water to exactly 20 cc. This was divided into two equal portions. The remainder of the procedure was the same as that employed for blood.

That this method yields accurate results was shown by the following experiments.

The urea content of the same samples of serum and whole blood was determined without preliminary treatment, and also by precip-

²² Marshall: this *Journal*, xiv, p. 283, 1913; xv, p. 287, 1913.

²³ *Ibid.*, xi, p. 527, 1912.

²⁴ *Ibid.*, xv, p. 493, 1913.

²⁵ In cases in which large amounts of urea were encountered an aliquot portion of this filtrate was used.

itating with alcohol as described for tissues. The results were exactly the same, as shown below. The results are expressed in milligrams of urea per 100 cc. of blood.

	WITHOUT PRELIMINARY TREATMENT	PRECIPITATION WITH ALCOHOL
Dog's serum.....	{ 22	22
	{ 21	21
Dog's serum.....	{ 29	28
	{ 28	29
Dog's blood.....	{ 28	
	{ 27	27
Pig's serum.....	14	14

That the urea is completely extracted by the procedure employed was also determined directly for the tissues by making an extraction in the usual way and then second and third extractions. This showed that even where extremely large amounts of urea are encountered 1 per cent or less of the total amount escapes extraction. Two cases are noted simply as an example, although the experiment was repeated a number of times.

10 grams of muscle yielded on first extraction 63.5 mgms. of urea, on second extraction 0.4 mgm. Ten grams of heart yielded on first extraction 61.5 mgm. of urea, on second extraction 0.6 mgm., and on the third 0.08 mgm. of urea.

The fact that additions of known amounts of urea to the alcoholic extract of tissues could be recovered quantitatively shows that, as with blood, no decomposition occurs on evaporation at a low temperature.

Unless otherwise specified, all urea injections have been made intravenously from a burette by a needle inserted directly through the skin, without exposing the vessel. This procedure is painless, and consequently no anaesthetic was used, except where indicated in the text. Specimens of blood were also drawn without exposing the vein, unless stated. Urine was collected in metabolism cages, and the collections completed by catheterization at the end of each stated period.

2. Urea content of the fluids and tissues of normal dogs.

The following table shows the amounts of urea present in the tissues and fluids of two normal dogs. The animals were selected after preliminary blood determinations had shown the blood urea to be normal. No attention was given to the diet although it might be stated that it was mixed, and not particularly rich in protein. The dogs were bled to death from the carotid artery, and specimens of the fluids and various organs secured immediately. D13 weighed 21.5 kilos, and D12, 11.3 kilos. Duplicate determinations were carried out on the materials from D13 whenever possible.

TISSUE OR FLUID	MILLIGRAMS UREA IN 100 GRAMS TISSUE OR 100 CC. FLUID		TISSUE OR FLUID	MILLIGRAMS OF UREA IN 100 GRAMS TISSUE OR 100 CC. FLUID	
	D13	D12		D13	D12
Blood.....	{ 28 27		Pancreas.....	{ 26 25	18
Blood serum.....	{ 29 29	22 21	Intestinal mucosa.....	{ 30 29	24
Bile.....	32		Parotids.....	29	21
Cerebrospinal fluid.....	25	21	Thyroid.....	30	37*
Liver.....	{ 32 23	25	Omentum.....	{ 5 5	6
Muscle.....	{ 25 25	18	Lymph glands.....		23
Heart.....	{ 28 30	22	Eye.....		17
Brain.....	{ 28 28	20	Spinal cord.....		17
Lung.....	{ 31 33	22	Testicles.....	{ 30 32	21
Spleen.....	{ 28 29	20	Prostate and urethra...		52
			Bladder.....		164
			Kidney.....	{ 183 159	221
			Urine.....	1640	

* Only 1-2 grams of this tissue were available for analysis.

The above data show that the percentage of urea in all the tissues and body fluids examined, except fat and the urinary apparatus, is approximately the same. The high figures for the kidney, bladder, and prostate are explained by the unavoidable contamination of these organs with urine. The urine examined was obtained from the bladder at autopsy. It is about fifty times

as concentrated as the blood and tissues in urea, and hence only a small amount would cause the noted increases. The concentration of urea in the kidney cells themselves, independent of the urine necessarily present in the organ, may or may not be markedly higher than the other tissues.

The low values obtained for fat are not surprising on account of the slight solubility of urea in fats, and also the small amount of water present in adipose tissue.²⁶ However, it was thought interesting to ascertain whether the fat interfered in any way with the estimation of urea.

Two equal samples of adipose tissue were obtained from a dog. To one of them were added 20 mgm. of urea dissolved in 10 cc. of a mixture of equal parts chloroform and alcohol. To the other, 10 cc. of the mixture without urea were added. Determinations were conducted in the usual way. The results are given below, together with a determination of the urea content of the spleen of the same animal.

10 grams of fat + 20 mgm. of urea yielded.....	21.0 mgm. urea
10 grams of fat alone yielded.....	1.3 mgm. urea
10 grams of spleen yielded.....	5.9 mgm. urea
This shows a recovery of 19.7 mgm. of the 20 mgm. added.	

The slight differences noted in the table between the various other tissues may or may not be real differences. Nencki and Sumanowsky and also Engels²⁷ have shown that the water content of the same tissues varies considerably in different dogs. Rumpf²⁸ in his tables shows variations in the fat and dry substance as well as water of certain organs. The heart and brain are high in fat, while the spleen is low. He further states that there is much variation in presumably normal organs. These facts, with errors incidental to the sampling and weighing of the moist tissues, which lose weight readily by drying, may readily explain the variations. However, whether the tissues contain exactly the same amount of urea per unit of weight or not is a question of no practical importance. The agreement between the figures representing the concentration of the urea in the blood and the tissues is rather striking. However, the blood analyses are calculated on a 100-cc. and the tissues on a 100-gram basis. Recalculating the

²⁶ Nencki and Sumanowsky: *Arch. f. exp. Path. u. Pharm.*, xxxiv, p. 313, 1894.

²⁷ *Ibid.*, li, p. 346, 1904.

²⁸ *Munch. med. Wochenschr.*, lii, p. 393, 1905.

blood or serum to a 100-gram basis, we would obtain figures slightly less; for D13, blood 26 mgm. serum 27 mgm. In this case also a sample of blood was weighed and the urea per 100 grams determined to be 25 mgm. In this connection, it will be well to mention a point, concerning most of the analyses of tissues reported further on in this paper. Blood serum was used in determining the concentration of urea in the blood in most cases. Undoubtedly some slight difference is encountered depending on whether the analyses are made on whole blood or serum, and it appeared that the serum yields slightly higher results.²⁹

3. Blood and tissue analyses before and after injection of urea.

As the most direct manner of approach to the problem of the disposition of excessive amounts of urea by the body, the following experiment was performed for the purpose of studying the blood and tissues of the same animal before and after injection of a large quantity of urea.

EXPERIMENT 1. Dog D4. Female terrier in good condition, weighing 4 kilos. The animal was etherized, and specimens of blood, liver, and skeletal muscle removed. Immediately afterward, 20 grams of urea dissolved in 75 cc. of distilled water were injected into the external jugular vein during seven minutes. One hour after the end of the injection, a specimen of blood was drawn. Four hours after the injection, the dog was bled to death from the carotid artery, and specimens of blood and tissues taken for analysis.

340 cc. of urine containing 3.79 grams of urea were excreted in the twenty-four hours previous to the experiment. 181 cc. were excreted during the experiment, containing 3 grams of urea. The urea content of the blood serum one hour after the injection amounted to 620 mgms. per 100 cc.

TISSUE	MILLIGRAMS OF UREA PER 100 GRAMS TISSUE BE- FORE INJECTION	MILLIGRAMS OF UREA PER 100 GRAMS TISSUE AT DEATH
Blood serum.....	21	493
Liver.....	22	351
Muscle.....	24	377
Spleen.....		409
Heart.....		367
Brain.....		397

²⁹ These slight differences would have no importance in clinical estimations of blood urea for purposes of diagnosis of renal function. In this connection see: Aronssohn: *Compt. rend. soc. biol.*, lxxi, p. 346, 1911; Javal: *ibid.*, p. 399; and Widal: *ibid.*, p. 492.

Before injection, this dog corresponded exactly with the control animals. Assuming the blood to be 7 per cent of the body weight, or 200 grams, it will be seen that at the end of an hour, but 1.7 grams of the 20 grams of urea injected remained in the circulation. In other words, ignoring for the moment the amount excreted by the kidneys, 18.5 grams, or about 93 per cent of the urea had found lodgment in the tissues. Three hours later the blood had fallen to 493 mgm. per 100 cc. accounting for 1 gram in the circulation.

4. Repeated injection of ascending doses.

In order to study the ability of dogs to excrete urea, and also to determine approximately the lethal concentration of urea in the body, repeated doses of ascending amounts of urea were given to a dog on successive days, with the idea of bringing about storage of that substance, if the excretory powers of the kidney could be exceeded. Occasionally the doses were interrupted to observe the time necessary to free the body from excess of urea.

EXPERIMENT 2. Dog D5, small female, weight 2.7 kilos. Preliminary specimen of blood showed normal urea content of 21 mgm. per 100 cc. serum.

DAY (Jan. 1914)	MILLIGRAM UREA IN 100 CC. SERUM	URINE UREA	REMARKS
		<i>grams</i>	
8	21		7.864 gms. urea intravenously.
9		(Estimated) 12.5	10 gms. urea intravenously.
10		14.38	10 gms. urea intravenously.
11		15.63	No urea.
12			10 gms. urea intravenously.
13		22.08	No urea.
14	46		No urea.
15		5.9	15 gms. urea intravenously.
16		Lost (estimated) 20.5	15 gms. urea intravenously.
17			No urea.
18		32.7	No urea.
19	21		No urea.
20		5.43	20 gms. urea intravenously (anaesthesia).
21		25.38	25 gms. urea intravenously, death.
Total....		157.20	112.86 gms. given.

The earlier injections of 10 grams of urea gave rise to no symptoms beyond loss of appetite for an hour or two after injection. When 15 grams were infused, January 15, the dog vomited twice, and exhibited great restlessness, moving constantly about his cage. The second 15 grams, however, caused no such symptoms. Anaesthesia was used for the 30-grams infusion. On January 21, the animal vomited when 18 grams had been run in. At 22 grams, he went into opisthotonic convulsion, and was thenceforth comatose, with continuous severe convulsive movements and labored breathing. Death occurred about fifty minutes after completion of the injection. At autopsy, the tissues appeared normal, except the brain, which was wet and hyperemic. The blood clotted readily. Specimens were saved for analysis.

Wherever the urinary values have been estimated, a daily excretion of 5.5 grams, in view of the figures for January 15 and 20, has been assumed. The figures for January 10 and 11 show that 10 grams of urea when injected are excreted in twenty-four hours. Including these estimations, approximately 157 grams of urea were put out during the experiment. 112 grams were injected. If to the latter figure is added the estimated formation in the body during thirteen days of 71.5 grams, one has a total of 83.5 grams. This is an excess of about 27.3 grams over the amount excreted, and one would expect to find approximately this amount in the body.

From the analyses of the autopsy specimens, the following figures were obtained.³⁰

Tissue	Urea milligrams per 100 grams or cc.
Blood (serum).....	1065(1034)
Liver.....	930
Heart.....	955
Brain.....	923
Spleen.....	1045
Lung.....	955
Muscle.....	914

³⁰ The figure given in parentheses in this and the following tables is the calculated urea content of 100 grams of serum or blood, assuming the specific gravity of serum to be 1.03, and of blood 1.06. The actual figure, however, is undoubtedly somewhat inconstant.

Here again, the content of the various organs is approximately the same, although at a high level. Taking 950 mgm. per 100 as the mean figure, the content of the entire body of the animal, whose weight was 2.7 kilos, was 25.9 grams, a very close approximation to the calculated amount given. While these figures are fairly rough, they show that it is possible to follow the movements of urea in the body closely, and indicate no conversion of urea into other substances.

This experiment, then, shows that a dog weighing approximately 2.7 kilos can rid himself of 10 grams of urea, injected intravenously, within twenty-four hours, and that 25 grams of urea given, as above, is a lethal dose for such an animal.

5. Analyses of blood and tissues of dogs with kidney involvement.

In this connection we cite two experiments on the injection of urea after ligation of one or both ureters, and also analyses of the blood and tissues of a dog with experimental hydronephrosis.

EXPERIMENT 3. Dog D9. Small male weighing 4.6 kilos, in rather poor condition, suspected of distemper. Fifteen minutes after ligating the right ureter, 12 grams of urea dissolved in 60 cc. of distilled water were injected into the external jugular vein, the injection occupying ten minutes. The animal showed no marked effects and was killed by bleeding twenty-three hours later. The bile and urine were obtained from the gall bladder and bladder at autopsy.

Tissue or fluid	Milligrams of urea per 100 grams tissue or 100 cc. fluid
Blood serum.....	93(90)
Bile.....	75
Liver.....	78
Right kidney.....	128
Left kidney.....	397
Urine.....	7010

EXPERIMENT 4. Dog D10. Small male Irish terrier weighing 5.8 kilos, recently recovered from an attack of distemper. Fifteen minutes after drawing specimen of blood, and ligating both ureters, 20 grams of urea dissolved in 70 cc. of distilled water were injected into the jugular vein during ten minutes. After forty-eight hours the dog seemed very sluggish; was able to walk, but hind legs were very spastic and tremulous. No spontaneous convulsions. Preferred to lie down. Etherized and killed by bleeding from carotid artery.

Autopsy. No pneumonia. Heart, liver and spleen appear normal. Intestines: Mucosa pale, normal amount of contents, somewhat excessive amount of bile, colon shows no hyperemia. Gall bladder distended with brown viscid bile. Pancreas slightly oedematous. Kidneys are markedly larger than when operation was performed. Ureters are both tied, greatly distended above the ligatures. When capsule of kidney is incised, large quantities of clear fluid well forth. The capsule strips readily, leaving smooth, pale, yellowish surface. Vessels are full of clear fluid, apparently replacing the blood. On section as soon as pelvis is cut urine spurts out under very high pressure. Pelvis enlarged, substance of kidney very oedematous. The fluid which exudes therefrom coagulates on standing. The cortex is greatly thickened. The ureters above the ligatures are injected and hemorrhagic. The above picture in kidneys is in sharp contrast to picture seen in ordinary ureteral ligations.

Tissue or fluid	Milligrams of urea per 100 grams tissue or 100 cc. fluid
Blood serum.....	827(803)
Bile	788
Liver.....	762
Pancreas	750
Muscle.....	768
Kidney.....	963

EXPERIMENT 5. K1. This dog was obtained from Dr. N. M. Keith. A band had been placed on the right ureter, not completely occluding it, but causing a large hydronephrosis. Twenty-one days later a similar band was placed on the left ureter, after which very little urine was secreted. Symptoms of uremia were marked, the dog passing into coma. Killed by bleeding four days after second operation.

Tissue	Milligrams of urea per 100 grams tissue or 100 cc. fluid
Blood	238(225)
Blood serum.....	272(264)
Liver.....	223
Heart.....	235
Muscle	205

Roughly speaking, the amount of urea which can be calculated to be present in the bodies of these animals corresponds with the amount one would expect from the daily formation of urea, according to body-weight, since the suppression of kidney function.

In the first dog, a single kidney was able to eliminate the urea fairly satisfactorily. In addition this dog was febrile, which condition itself brings about a rise in the blood urea.³¹

³¹ Our own observations; and see also Gscheidlen: *Studien über den Ursprung des Harnstoffs*, Leipzig, 1871.

The anatomical picture seen in these dogs is worthy of notice. The obstructed kidneys are tremendously enlarged and oedematous and the urine in the pelvis is under extremely high pressure. Active secretion has gone on in spite of the obstruction, and the tendency to suppression often seen after manipulations of the ureter overcome.

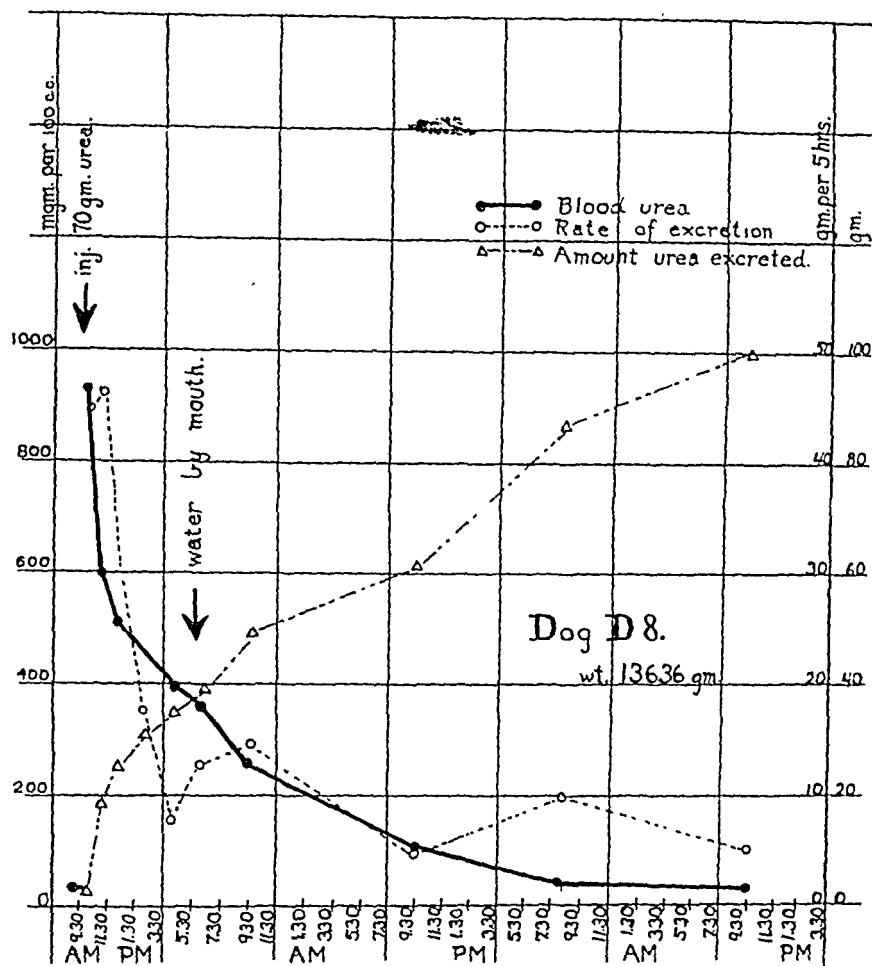
6. Single large injections.

To study the elimination of urea from the body, single large intravenous injections were given to dogs, and estimations of the blood urea and of the amount of urea excreted in the urine made at frequent intervals thereafter, until normal conditions were reestablished.

EXPERIMENT 6. Dog D8, large black female, weight 13.6 kilos. A preliminary specimen of blood showed a normal urea content of 28 mgm. per 100 cc. At 9.16 a.m., 70 grams of urea dissolved in 155 cc. of distilled water were injected in the external jugular vein during fifteen minutes; specimens as follows:

DAY (Feb. 1914)	HOUR	MILLIGRAMS OF UREA PER 100 CC. BLOOD SERUM	GRAMS OF UREA IN URINE
8	9.05 a.m.	28	
	9.31	Injection	
	9.33	915	
	9.36		2.94
	11.00	600	14.42
	12.00	531	5.99
	2.00 p.m.	lost	7.02
	4.00	396	2.97
	6.00	360	5.12
	9.30	258	9.99
9	10.15 a.m.	100	11.92
10	8.00 p.m.	39	24.57
	10.00 a.m.	28	13.17
	Total.....		99.91

Immediately after the injection the animal seemed dazed and unable to stand up, but did not lose consciousness or have any convulsions; vomited once or twice. At about 6 p.m. the first day, was given water, which was eagerly taken. In twenty-four hours, seemed quite normal.



CURVE I. Excretion of urea after single large injection.

The findings in this dog have been expressed as curves in the accompanying figure. The blood urea falls extremely rapidly at first—due no doubt to more complete diffusion into the tissues—then less rapidly, and at the end approaches the normal level very slowly. The rapid elimination of urea is accompanied by a characteristic diuresis, which decreases with the concentration of urea in the blood. The rate of excretion of urea (calculated in the figure, to give a sharper curve, as for a 5-hour period) also falls rapidly. That it is not independent of the urinary conditions is well shown in this figure when, after giving the animal a large quantity of water, the diuresis increased, the rate of elimination rose markedly,

the total excretion was augmented, and the fall in blood urea became more rapid. The rise in the rate of excretion at the thirty-sixth hour is no doubt part of the regular diurnal variation (see next experiment).

EXPERIMENT 7. Dog D11, small female, weight 6.8 kilos, in excellent condition. In order to be able to estimate closely that proportion of the excreted urea due to the normal urea formation within the animal, this dog was placed upon a constant diet, moderate in proteins, of dog biscuit, milk, and water, and daily estimations of the urea output were carried on over a long period. Figures for a week typical of these analyses are given below: On March 14, 10 grams of urea were given intravenously.

	MARCH						
	8	9	10	11, 12	13	14	15
Grams urea.....	5.51	5.88	5.32	10.37	6.15	15.36	5.40

The figures for March 14 show that 10 grams of urea given intravenously can be completely excreted by a dog of this size within twenty-four hours. The average daily output—excluding March 14—is 5.52 grams.

On March 23 a number of analyses were carried out on catheterized specimens to ascertain if the urea output was constant at all times in the twenty-four hours.

TIME	GRAMS UREA EXCRETED	GRAMS UREA CALCULATED PER HOUR
11.30-12.30 p.m.	0.33	0.33
12.30- 2.00 p.m.	0.47	0.31
2.00- 3.30 p.m.	0.51	0.34
3.30- 5.30 p.m.	0.86	0.43
5.30-11.30 p.m.	1.63	0.27
11.30-11.30 a.m.	1.88	0.15
Total.....	5.68	

This table shows a markedly greater output of urea, per unit of time, during the day than during the night. The dog received no food after 3.30 p.m.

On March 25, 20 grams of urea dissolved in 65 cc. of distilled water were given in the external jugular. The injection occupied eleven minutes (10.41-10.52 a.m.). Specimens as follows:

DAY (March, 1914)	HOUR	MILLIGRAMS UREA PER 100 CC. OF BLOOD	GRAMS UREA IN URINE
25	10.55 a.m.	505	
	11.00	422	1.00
	11.30	395	1.14
	1.00 p.m.	310	3.96
	3.00	212	4.94
	5.00	156	3.06
	11.00	97	4.23
26	11.00 a.m.	47	4.04
27	11.00 a.m.	25	8.78
28	11.00 a.m.	23	5.47
30	11.00 a.m.	23	

After the injection, the dog appeared only slightly depressed, but later vomited and defecated two or three times. For twenty-four hours very little was eaten, but after that time the animal appeared to be normal.

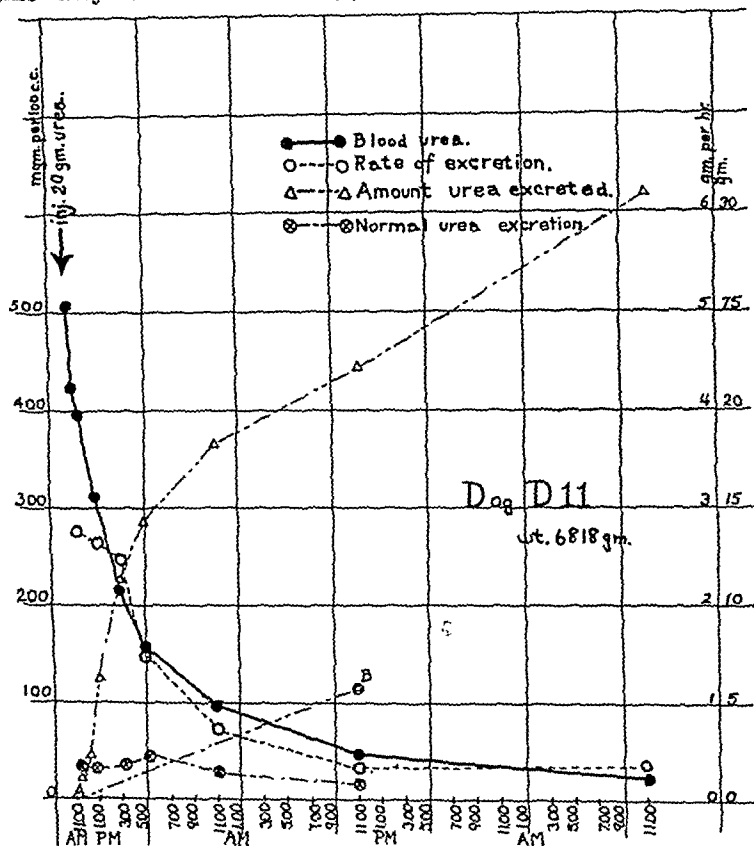
The results of this experiment are also expressed as curves in the accompanying figure.

In this experiment, the irregularities seen in Curve I were avoided by withholding water during the first twenty-four hours. Aside from this, the results are essentially the same. As the blood urea approaches the normal limit, its fall becomes very slow. The rate of excretion appears to remain more nearly constant while the blood urea is high, and later falls. The normal rate of excretion, previously determined, is included in this figure. The line AB represents the amount of urea normally excreted in twenty-four hours. The same rapid fall of the blood urea during the first few minutes of the experiment, due to absorption by the tissues, is seen in this experiment. It represents, however, only the later stages of this absorption, since, although the first specimen of blood was drawn as soon as possible after the end of the injection, a simple calculation shows that only about 2.4 grams remained in the blood stream; *i.e.*, 83 per cent of the injected urea had already left the circulation.

The amount of urea excreted during the first twenty-four hours, 22.37 grams, shows, if we assume 5.52 grams of urea to have been formed in the body during the day, that 3.15 grams of the injected urea still remain in the body. This is accounted for on the second day when 8.78 grams, an excess of 3.46 grams over the normal figure, were put out. But, calculating from the content of the

blood at 11 a.m. March 26 one finds that there are presumably in the animal at this time only, roughly, 3.2 grams of urea; all of which, in addition to a normal daily quantity, is put out on March 26-27. One is therefore drawn toward the opinion that the formation of urea within the body was lessened on March 25-26.

It must be borne in mind that these figures are only approximate, since they take no account of (1) excretion of urea by other means



CURVE II. Excretion of urea after single large injection.

than the urine, as the bile, or (2) of any modification of the general protein metabolism caused by urea injections.³²

³² Heilner: *Zeitschr. f. Biol.*, lii, p. 216, 1909; see also the experiments with feeding of NH_3 and urea; Grafe: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 1, 1912; lxxviii, p. 347, 1912; lxxxiii, p. 25, 1913; Abderhalden: *ibid.*, lxxviii, p. 1, 1912; lxxxiii, p. 338, 1913; and various articles by both in lxxxiv, 1913.

7. *Repeated small injections.*

When animals die after large doses of urea, they usually do so from one to two hours after cessation of the injection, being in the meantime comatose, with rapid respiration and convulsions. In the following experiment, therefore, it was considered that the onset of severe convulsions was an indication of marked intoxication.

EXPERIMENT 8. Dog D7. Female, weight 9.1 kilos. After a preliminary blood analysis, the animal was anaesthetized, cannulas inserted in the jugular vein and the carotid artery, and injection made of a solution of urea in distilled water containing 40 grams per 100 cc., through the venous cannula. After each 10 grams of urea had been infused the inflow was stopped, and blood drawn three minutes later from the artery. This was continued until 100 grams had been introduced, when the ether cone was removed. After noting the symptoms for fifteen minutes, the animal was bled to death.

TIME	UREA INJECTED, PER PERIOD	TOTAL UREA INJECTED	MILLIGRAMS OF UREA PER 100 CC. BLOOD SERUM	AMOUNT UREA IN BLOOD, CAL- CULATED	UREA REMOVED FROM BLOOD IN 3 MIN.	PERCENT OF INJECTED UREA RE- MAINING IN BLOOD
	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>grams</i>	
4.23			29			
4.28	10	10				
4.31			310	1.970	8.114	18.8
4.36	10	20				
4.39			483	3.072	8.898	11.0
4.44	10	30				
4.47			609	3.873	9.139	8.6
4.55	10	40				
4.58			776	4.935	8.938	10.0
5.05	10	50				
5.10			940	6.016	8.919	10.8
5.16	10	60				
5.19			1104	7.021	8.995	10.0
5.24	10	70				
5.27			1291	8.211	8.811	11.8
5.34	10	80				
5.37			1450	9.222	8.989	10.1
5.41	10	90				
5.44			1608	10.227	8.995	10.0
5.48	10	100				
5.51			1692	10.761	9.400	6.0
Total.....		100			89.198	

In calculating the figures for this table, the usual assumption was made, that the blood made up 7 per cent of the bodyweight. It will be seen that the tissues take up with great regularity about 90 per cent of the amount of urea injected within three minutes after the injection, whether the concentration in the body be high or low. Perfect isotonicity is probably not reached in three minutes, however, since if the blood urea at the end (1.692 grams per 100) be multiplied by the body-weight, the result will be in excess of the actual amount present.

About fifteen minutes after the last injection, the animal was sacrificed, and specimens of the organs taken for analysis. The results follow:

Tissue	Urea milligrams per 100 grams
Brain.....	1182
Liver.....	1215
Muscle.....	1254
Lung.....	1335

The urea content of the organs is distinctly less than that of the blood serum. It should be noted that fifteen minutes elapsed after the last sample of blood was drawn before the animal was killed.

The urine secreted during the experiment measured 265 cc. and contained 8.8 grams of urea.

Symptoms were noted as follows: After the injection of 30 grams, flush over belly; after 50 grams, slight tremors of legs; after 80 grams, tremors more pronounced, after 90 grams, opisthotonic convulsion. As soon as the ether cone was removed at the end of the experiment, the convulsions became very severe. Death by bleeding from the carotid. The brain appeared hyperemic, and there was an excess of fluid in the subarachnoid space.

8. Urea in human nephritic tissues.

The following figures have been obtained by analyses of tissues taken from human cases at autopsy. In some instances, the clinical findings ante-mortem are included, for which we are indebted to Dr. R. R. Snowden and Dr. F. H. Hinman.

CASE 1. Autopsy 4034. Acute nephritis, pyelitis, cystitis, lobar pneumonia, pericarditis. Muscle 134 mgm. urea per 100 grams.

CASE 2. Autopsy 4056. Vesical calculi and enlarged prostate, dilatation of bladder and ureters, acute and chronic nephritis, with abscesses. Blood urea shortly before death 212 mgm. per 100 cc. Phthalein 7 per cent.³³

Tissue	Milligrams urea per 100 grams
Muscle.....	260
Liver.....	322
Spleen.....	310

CASE 3. Autopsy 4062. Chronic diffuse nephritis, arteriosclerosis, encephalomalacia, psychosis. Phthalein one month before death, 21 per cent. Albumin and casts. B. P. 190 to 240.

Tissue	Milligrams urea per 100 grams or cc.
Blood serum.....	750(70S)
Liver.....	{ 603 617
Muscle.....	635
Spleen.....	660
Heart.....	621
Kidney.....	672

CASE 4. Autopsy 4064. Pulmonary induration, hypertrophy right heart, oedema, chronic passive congestion. One month before death phthalein 29 per cent in first hour, lactose delayed, blood urea 45 mgm. per 100 cc. Albumin and casts. B. P. 180-240.

Tissue	Milligrams urea per 100 grams or cc.
Blood.....	114(10S)
Muscle.....	110
Heart.....	112
Liver.....	115
Brain.....	102
Kidney.....	124

CASE 5. Autopsy 4072. Renal calculi, destruction of one kidney, chronic and acute nephritis in the other, bronchopneumonia.

Tissue	Milligrams urea per 100 grams or cc.
Spleen.....	576
Muscle.....	622
Liver.....	550

CASE 6. Autopsy 4089. Subacute diffuse nephritis, syphilis, bronchopneumonia. (Large white kidney). Two days before death, phthalein only in traces. One and one-half hour before death, blood urea 204 mgm. per 100 cc. Albumin and casts. B. P. 180-220.

³³ Rowntree and Geraghty: *Arch. of Int. Med.*, ix, p. 284, 1912.

Tissue	Milligrams urea per 100 grams
Blood serum.....	282(271)
Muscle.....	257
Liver.....	300
Spleen.....	269
Heart.....	279

In these tables, the figures correspond well with those given in the animal experiments. The lack of agreement sometimes noted may be attributed to the exigencies of obtaining specimens from the autopsy table—drying, etc. It is evident that in human beings also, accumulations of urea in the blood mean a corresponding increase in the tissues. One notes a well marked retention (Case 4) in a man without nephritis, but with severe chronic passive congestion. In cases 4 and 5 the figures for the kidney are important. They are scarcely higher than those for the other organs, and it would appear that the renal cells have lost their power to concentrate urea. In cases 3 and 5 the retention is surprisingly high.

The quantity of urea in the bodies of nephritics recently dead varies greatly. We cannot draw any general conclusions at present regarding its rôle in the causation of death. In all these cases, the nephritis was complicated with other conditions.

III. DISCUSSION.

In summarizing the results of our experiments, it will be seen that we have proved again, more conclusively than ever, that urea occurs in *all* the organs and tissues of the body. Further, we have shown that urea occurs throughout the body in approximately uniform concentration, except in the fat and in the urinary tract.

The high content of the kidney is to be explained by the presence of varying quantities of urine in the renal tubules. This is a fact that should be always borne in mind in kidney analyses for any substance. Concerning the bladder and prostate (which of course includes a portion of the urethra) from which all urine has been drained off, it would appear from the researches of Gerota,³¹ who showed absorption of urea from the bladder, and of Hambur-

³¹ *Arch. f. (Anat. u.) Physiol.*, 1897, p. 423.

burger³⁵ who demonstrated the ability of bladder epithelial cells to take up urea *in vitro*, that a certain amount of absorption from the urine has taken place.

This approximately uniform distribution of urea is also found when the urea content of the body has been increased, either by injections of urea, by interference with excretion by the kidneys, or by both means together. The body cells are able to take up in this manner very large quantities of urea; the greatest quantity injected by us, which gave a concentration in the tissues of 1200 mgms. per 100 grams, being taken up as readily as the smallest. Whenever the bile has been analyzed, it has appeared to have approximately the same concentration of urea as the blood—consequently when the blood urea is high, the bile may carry off sensible quantities of urea.

The diffusion of the urea to all parts of the body is accomplished very quickly. No matter how soon after the end of the injection blood is drawn, a large part of the urea has already gone out of the circulation. In general, the distribution of urea to all the tissues which take it up may be said to be complete within fifteen to eighteen minutes after the injection is finished. In this connection, a study of the figures will make it apparent that a certain proportion of the material comprising the body does not take up urea. This proportion is made up of the fat, bone, cartilage, teeth, outer layers of the skin, etc. In the case of the animal D4 there was roughly 0.9 gram of urea in the body at the beginning and 20 grams were injected. Of this 2.6 grams were excreted during the experiment, leaving 16.01 grams in the body. Since the average concentration in the tissues was 375 mgm. per 100 grams it follows that about 93 per cent of the tissues contained urea—granting that the distribution is uniform. Dog D7, calculated in like manner, gives a figure of 86 per cent. Dog D11, using the whole blood as the index, gives a figure of about 72 per cent one-half hour after the injection. This low percentage may be accounted for by the much larger amount of fat present in D11, which was a very well nourished animal.

The ability of the tissues to take up urea is not changed whether the concentration in the blood is high or low (D7), the same per-

³⁵ Hamburger: *Osmotische Druck und Ionenlehre*, Wiesbaden, 1902, Bd. I.

centage of the injected urea being removed from the circulation in three minutes in either case.

The ability of the kidneys to rid the body of urea is very great. After injection, the well known diuresis occurs, the quantity of urine secreted being in proportion to the amount of urea injected. The concentration of urea in this urine apparently does not rise beyond a certain level, 40 grams per liter being seldom exceeded by our dogs. The quantity of urea given out, however, may be enormous, secretion occurring in one dog (D8) at the rate of over 0.66 gram per kilo per hour, in another (D11) at the rate of 0.36 gram per kilo per hour, and in a third (D7) at the rate of 0.48 gram per kilo per hour, over short periods. These figures are much in excess of the maximum given by Ambard³⁶ and that they do not reach the limit is shown by a calculation from one of Herter's³⁷ tables, which gives an excretion of 1.185g rams per kilo per hour. As the blood urea falls, the rate of elimination of urea falls with it. It would appear, then, that if the percentage of urea in the blood rises from any cause, the kidney cells will be thereby stimulated, or at least allowed, to secrete increased amounts of urea. Then in nephritis with retention, the kidney cells remaining would excrete urea more rapidly, and one could imagine a kidney with much of its parenchyma replaced by scar tissue still keeping the blood urea constant, although at a higher level than normal, like an insufficient heart under the influence of digitalis. It would also explain the fall to normal of the blood urea observed by Folin³⁸ in nephritics placed on a low protein diet, which occurred even when there had been no progressive rise in blood urea theretofore. That an increase of function may not occur in this way when the renal epithelial cells are actually acutely damaged, is apparently indicated by the experiments of Herter³⁹ with canthardin nephritis.

After a certain amount of dehydration has occurred, elimination is retarded by lack of water for the kidneys to secrete. If water is then given, urea is again thrown out at an increased rate.

In our few experiments made on animals whose kidneys or ureters had been interfered with, the amount of urea which,

³⁶ *Compt. rend. soc. biol.*, lxxv, p. 712, 1908; lxxvi, p. 29, 1909.

³⁷ *Johns Hopkins Hospital Reports*, ix, p. 69, 1900.

³⁸ Folin, Denis and Seymour: *Arch. of Int. Med.*, xiii, p. 224, 1914.

³⁹ *Johns Hopkins Hospital Reports*, ix, p. 69, 1900.

according to calculation from the analyses, is in the body at the time of death, corresponds very well with the amount which one would expect to be formed in the animals, according to their weight, since the time of operation, plus any urea which was injected. This fact in connection with others which we have brought forward goes far toward explaining the phenomena observed in nephritis with decreased urea output. Since a man weighing 70 kilos has only 4-5 liters of blood, and may be assumed to form around 30 grams of urea per day, it is readily seen that any failure to eliminate would immediately give rise to overwhelming quantities of urea in the blood, did it all remain in the circulation. That it must be taken up by the tissues is the conclusion arrived at by practically all workers. Increased amounts of urea in the tissues in nephritis have been definitely shown, but until now there has been great confusion as to the quantity stored by them. Since, as we have shown, it is taken up equally by all tissues, the formation of 30 grams of urea in a day in a man of 70 kilos, even if none is excreted, will leave only about 2 grams in the blood, which is equivalent to a rise of about 0.4 gram per liter, or 40 mgm. per 100 cc. in the blood urea. At this rate, it would take five to six days of complete retention to raise the blood urea to 200 mgm. per 100 cc., a figure not infrequently occurring in nephritics. With partial excretion, and a low protein diet, this accumulation would be deferred much longer.

The moderate number of analyses we have made on the organs of nephritics coming to autopsy fall in perfectly with the results in the dog experiments.

In every case a rise in blood urea has been accompanied by a corresponding rise in the urea content of tissues, which in all tissues has been approximately the same. As a rule, this figure in fatal cases is found to lie near 200 mgm.⁴⁰ per 100 cc., though in two cases it reached the surprising levels of about 600 and 650 mgm. per 100 cc. In all of these cases other conditions besides nephritis were present, and must be reckoned with as factors in the fatal outcome.

⁴⁰ Widai: *Bull. et mém. d. hôp. Paris*, series 3, xxxii, p. 627, 1911; Rown-tree and Fitz: *Arch. of Int. Med.*, xi, p. 258, 1913; Folin and Denis: *This Journal*, xiv, p. 29, 1913.

The very general distribution of urea is entirely consonant with theoretical conclusions derived from diffusion studies on this substance. It is a non-conductor, ionizing very slightly, and it has been found⁴¹ to permeate readily through all kinds of cell membranes; so readily, in fact, that when cells are placed in a urea solution, the urea concentration within them becomes the same as that outside, instantly; or at least so soon that not the slightest effect upon the cell, due to any change in osmotic pressure, can be observed. Therefore urea is free to go, and go quickly, anywhere in the body, it can never cause hemolysis, it is most readily excreted by the normal kidney, and it is the most desirable form in which nitrogen could be put for elimination from the body.

Apparently, then, urea should be a non-toxic substance. Our experiments show that it is non-toxic in any moderate or fairly large amounts. Only when introduced in enormous doses—in the neighborhood of 1000 mgm. per 100 grams of body-weight, does it produce a fatal effect. However, in doses somewhat lower than this it does produce definite symptoms—peripheral dilatation, vomiting, muscular twitching, and convulsions, as noted by Herter. (Diarrhoea has not been a noteworthy symptom.) Therefore, while it is improbable that urea is the only toxic agent in uremia, it may take some share in producing the symptoms of this condition. In this connection, it should be remembered that when urea is increased in the blood, it is equally increased in the central nervous system and at all other points.

We have not found evidence of any increase of urea formation in our injected animals, to correspond with the increased nitrogen output observed by Heilner⁴² in rabbits made the subjects of subcutaneous infusions of urea. In D11 the average daily output of urea has remained quite constant, independent of the injections. It is true that there appears to have been a slight diminution in the formation on the day of the injection of 20 grams of urea, made up by a corresponding increase the day after, but no conclusions can be drawn from a single such observation.

We have found no evidence of the transformation of urea into other substances in the body.

⁴¹ Gryns: *Pflüger's Archiv*, lxiii, p. 86, 1896; also Hamburger: *Osmotische Druck u. Ionenlehre*, Bd. I and III.

⁴² Heilner: *Zeitschr. f. Biol.*, lii, p. 216, 1909.

Our methods of determining urea should obviously be of the greatest aid in perfusion experiments, on surviving organs, designed to show the seat of formation of urea. We are at present engaged in carrying out such experiments, and will shortly publish the results thereof.

IV. CONCLUSIONS.

The conclusions drawn from our experiments are as follows:

1. The method described in this paper gives accurate results in the analysis of tissues for urea.

2. Urea is present in all the organs and tissues of normal animals.

3. The urea content of all organs and tissues is approximately uniform, and approximately equal to that of the blood, both in normal conditions and when there is an abnormally large amount of urea present. Exceptions to this rule are fat, which has a low content, and the urinary tract, which has a high content.

4. When urea in solution is injected intravenously, it diffuses to all parts of the body almost instantly, the diffusion being complete in a few minutes.

5. Urea is eliminated very rapidly by the kidneys; the rate of excretion may rise to 16 grams per kilo of body weight per day, or much higher.

6. The rate of excretion of urea in normal animals is directly proportional to the concentration of urea in the blood; it may be retarded, however, by dehydration of the organism.

7. When excretion of urea is prevented, the entire amount formed is stored in the body—except small amounts secreted in the bile, sweat, etc.—and there is no evidence of the conversion of urea into other substances.

THE CHEMISTRY OF GLUCONEOGENESIS.

VIII. THE VELOCITY OF FORMATION AND ELIMINATION OF GLUCOSE BY DIABETIC ANIMALS.¹

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(Received for publication, April 15, 1914.)

In the study of the glucogenetic properties of the fatty acids and their derivatives, it was frequently observed that after subcutaneous or oral administration of the substance, the complete yield of "extra" glucose was not obtained within the first period of twelve hours. This retardation in the appearance in the urine of the "extra" glucose may be due to one of the following causes: 1. Slow absorption of the substance fed. 2. Slow rate of the glucose formation. 3. Slow elimination of the glucose by the kidneys. It was with a view to determining which of these factors was the underlying cause that these experiments were undertaken.

To eliminate the factor of absorption the substances were administered intravenously (saphenous vein) and the nitrogen and glucose in the urine were determined in short periods of two or three hours, as indicated in the tables. The "extra" glucose eliminated was computed by assuming the mean D:N ratio of the fore and after periods of twelve hours.

The animals were phlorhizinized by the daily injection of 1 gram of phlorhizin ground up in olive oil. The urine was collected by catheter and the bladder washed thoroughly with distilled water at the end of each period. This is very important when the periods are short.

In Experiment XXXIV, 7.4 grams ($\frac{M}{10}$) of propionic acid, neutralized with sodium hydroxide, were dissolved in 150 cc. of isotonic salt solution and slowly injected intravenously at the beginning of period B. No anesthesia was found necessary for these operations.

¹ Aided by a grant from the Rockefeller Institute for Medical Research.

The D:N ratio in the fore period of twelve hours was 2.62 and in the after period was 3.04; we therefore assume the mean of these ratios, 2.83, as the one which would have persisted if no propionic acid had been administered. This assumption is borne out by the fact that the D:N ratio in the one-hour fore period (A) was 2.76. By multiplying the nitrogen of each period by 2.83 we found the amount of glucose that originated from the catabolized protein. And by subtracting this figure from the total glucose, the amount of "extra" glucose was obtained.

During period B, the first two hours after the propionic acid injection, 2.34 grams of "extra" glucose were eliminated. In period D there was a considerable drop in the amount of "extra" glucose elimination, amounting to only 0.96 gram, and in periods E and F, 0.83 and 0.82 gram were eliminated respectively. The total amount of "extra" glucose eliminated in the eleven hours was 7.83 grams. This is considerably less than was found previously² after subcutaneous or oral administration. The difference may be explained by the rapid entrance of propionic acid into the blood and the excretion of some by the kidneys before it is converted into glucose.

In Experiment XXXV a similar amount of propionic acid was similarly injected intravenously at the end of the first hour of period A. The D:N ratios of the fore and after periods of twelve hours were 2.32 and 2.91. In the calculation of the "extra" glucose we assumed the mean of the two, which is 2.61. The correctness of this assumption is again borne out by the fact that the D:N ratios in periods E and F were 2.53 and 2.51 respectively.

During the second hour of period A, *i.e.*, the first hour after the propionic acid administration, there was an elimination of 0.25 gram of "extra" glucose. In period B 1.94 grams and in period C 2.66 grams were eliminated. In period D there was a marked decline; only 0.84 gram was eliminated. The total amount of "extra" glucose eliminated was 5.69 grams.

In Experiment XXXVI, 9 grams ($\frac{M}{20}$) of glucose were dissolved in 150 cc. of isotonic salt solution and injected intravenously at the end of the first hour of period A. The D:N ratios of the fore and after periods were 3.23 and 3.77 respectively. The mean of

² Ringer: this *Journal*, xii, p. 511, 1912.

3.55 was used in computing the "extra" glucose. During the second hour of period A, *i.e.*, the first hour after the glucose administration, 3.91 grams of extra glucose were eliminated. In period B 2.47 grams of "extra" glucose were eliminated and in period C there was 0.39 gram of "extra" glucose. The total amount of "extra" glucose eliminated was 6.77 grams.

In experiment XXXVII, 9 grams of glucose ($\frac{M}{20}$) dissolved in about 50 cc. of water were given *per os* at the beginning of period B. During the first two hours (period B) 5.32 grams of "extra" glucose were eliminated, and 2.34 grams were eliminated in period C. In periods D and E 0.68 and 0.37 grams of "extra" glucose were eliminated. The total elimination of "extra" glucose was 8.71 grams.

Discussion of results.

The curves showing the velocity of the "extra" glucose elimination are plotted for the purpose of bringing out more clearly the results of these experiments. They show with what explosive rapidity the glucose is excreted by the kidneys after it enters the blood stream. The absorption of the glucose from the gastrointestinal tract, in Experiment XXXVII, must be exceedingly rapid, for the curve during the first two hours scarcely differs from that of Experiment XXXVI.

The curves of "extra" glucose elimination, after glucose administration, are very typical and differ materially from those which follow propionic acid administration. The former passes the 5-gram mark in less than two hours, whereas the corresponding point on the propionic acid curve is touched in four to five hours. Since factors of absorption do not play any rôle here, *the difference in the time relationship can be attributed only to the time required for the synthesis of glucose from propionic acid.*

These experiments justify the conclusions that:

1. There is no delay in glucose elimination by the kidneys, but it is excreted almost as fast as it enters the blood stream.
2. In the slow elimination of "extra" glucose, factors of absorption and velocity of sugar formation may play contributing rôles.

Experiment XXXIV. Dog's weight 10.85 kgm.

PERIOD	NUMBER OF HOURS	NITROGEN	NITROGEN PER HOUR	GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
VI	12	3.46	0.288	9.07	0.756	2.62		7.4 grams of propionic acid as sodium salt, dissolved in 150 cc. of isotonic salt solution, injected intravenously at the beginning of period B.
VII A	1	0.36	0.360	0.993	0.993	2.76		
B	2	0.716	0.358	4.370	2.185	6.10	2.34	
C	2	0.860	0.430	5.31	2.650	6.18	2.88	
D	2	0.723	0.362	3.01	1.505	4.16	0.96	
E	2	0.567	0.284	2.43	1.215	4.29	0.83	
F	3	0.690	0.230	2.77	0.925	4.02	0.82	
Total for per. VII		3.916		18.883		4.81	7.83	
VIII	12	2.79	0.232	8.52	0.710	3.04		

Experiment XXXV. Dog's weight 6.80 kgm.

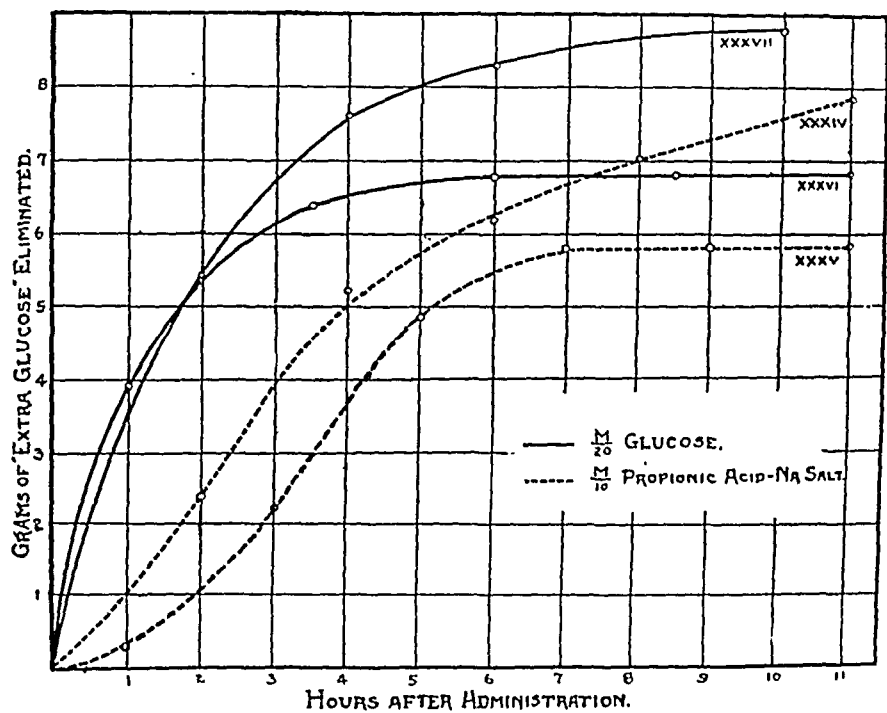
PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
X	12	4.050	0.337	9.37	0.781	2.32		At end of first hour of period A 7.4 grams of propionic acid as sodium salt dissolved in 150 cc. of isotonic salt solution, injected intravenously.
XI A	2	0.684	0.342	2.03		2.98	0.25	
B	2	0.245	0.122	2.58	1.290	10.54	1.94	
C	2	0.668	0.334	4.40	2.200	6.57	2.66	
D	2	0.968	0.484	3.37	1.685	3.49	0.84	
E	2	0.802	0.401	2.03	1.015	2.53		
F	2	0.704	0.352	1.759	0.879	2.51		
Total for per. XI		4.071		16.169		3.97	5.69	
XII	12	3.01	0.251	8.75	0.729	2.91		

Experiment XXXVI. Dog's weight 10.49 kgm.

PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
X	12	3.04	0.253	9.83	0.819	3.23		At end of first hour of period A, 9 grams of glucose dissolved in 150 cc. of isotonic salt solution, injected intravenously.
XI A	2	0.608	0.304	6.04		9.94	3.91	
B	2.5	0.717	0.287	4.98	1.990	6.95	2.47	
C	2.5	0.558	0.223	2.34	0.936	4.20	0.39	
D	2.5	0.564	0.225	1.95	0.783	3.47		
E	2.5	0.575	0.230	2.07	0.828	3.60		
Total for per. XI		3.022		17.38		5.76	6.77	
XII	12	2.68	0.223	10.09	0.841	3.77		

Experiment XXXVII. Dog's weight 9.48 kgm.

PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
I	12	5.05	0.420	16.28	1.36	3.22		9 grams of glucose dissolved in about 50 cc. of water given <i>per os</i> at beginning of period B.
II	12	4.65	0.387	14.50	1.21	3.12		
III A	2	0.614	0.322	2.280	1.140	3.72		
B	2	0.728	0.364	7.616	3.808	10.48	5.32	
C	2	0.583	0.291	4.176	2.088	7.17	2.34	
D	2	0.668	0.334	2.789	1.394	4.19	0.68	
E	4	1.270	0.318	4.376	1.094	3.44	0.37	
Total for per. III		3.86		21.237		5.50	8.71	
IV	12	3.41	0.284	10.90	0.909	3.20		
V	12	3.70	0.308	11.66	0.972	3.15		



STUDIES IN CARBOHYDRATE METABOLISM.

VI. THE INFLUENCE OF THYREOPARATHYROIDECTOMY UPON THE SUGAR CONTENT OF THE BLOOD AND THE GLYCOGEN CONTENT OF THE LIVER.

BY FRANK P. UNDERHILL AND NORMAN R. BLATHERWICK.

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(Received for publication, April 16, 1914.)

It is generally recognized that the thyroid-parathyroid mechanism bears a close relationship to carbohydrate metabolism although current ideas concerning this correlation are somewhat indefinite and vague. From the experimental standpoint it seems probable that the parathyroids exercise a more profound function in regulating carbohydrate transformations than do the thyroids. In support of this statement may be quoted, as examples, the work of Hirsch,¹ Eppinger, Falta and Rudinger,² Pari,³ and Underhill with Saiki⁴ and Hilditch,⁵ who have demonstrated the following facts. After thyreoparathyroidectomy the assimilation limit for dextrose given by mouth or subcutaneously is significantly lowered in dogs. Thyroidectomy alone fails to produce such an effect. On the other hand, the extirpation of three parathyroids causes a diminished assimilative power for dextrose, and the removal of three parathyroids plus one thyroid produces no greater effect than is observed after the excision of the three parathyroids alone. With two intact parathyroids the power of the body to utilize sugar is not decreased even in the complete absence of the thyroids.

¹ Hirsch: *Zeitschr. f. exp. Path. u. Ther.*, iii, p. 393, 1906; *ibid.*, v, p. 33, 1908.

² Eppinger, Falta and Rudinger: *Wien. klin. Wochenschr.*, 1908, p. 241; *Zeitschr. f. klin. Med.*, lxvi, p. 1, 1908; lxvii, p. 1, 1909.

³ Pari: *Biochem. Zeitschr.*, xiii, p. 281, 1908.

⁴ Underhill and Saiki: *this Journal*, v, p. 225, 1908.

⁵ Underhill and Hilditch: *Amer. Journ. of Physiol.*, xxv, p. 66, 1909.

It is the purpose of the present paper to report some experiments upon dogs the results of which demonstrate in a more definite manner than has been previously recorded that the thyroid-parathyroid mechanism stands in an intimate connection with carbohydrate metabolism. It is also indicated that the parathyroids are the active agents of the mechanism under discussion.

Our experiments show that during the tetany which develops after thyreoparathyroidectomy *glycogen entirely disappears from the liver and blood sugar content is markedly lowered or may even be reduced to zero*. It is also indicated that *this action may be ascribed to the lack of parathyroid tissue since the phenomena may be observed when all the parathyroids are removed and some thyroid tissue remains*. The reverse operation, that of removing the thyroids and leaving the parathyroids intact has not been carried through in the present investigation since there is little to indicate that any abnormal effect would be produced inasmuch as dogs in this laboratory have lived with four parathyroids only for two years without visible metabolic disturbances.

The data yielded after thyreoparathyroidectomy resemble closely those obtained as a result of phosphorus⁶ and hydrazine poisoning.⁷

EXPERIMENTAL.

The influence of thyreoparathyroidectomy.

Experiment 1. From a full-grown mongrel bitch of 8.5 kilos the thyroids and parathyroids were removed under ether anaesthesia. The animal made a rapid recovery and on the following day seemed normal. No food was given. The next day she seemed quiet and developed an unusual thirst. Tremors of the hind legs were first observed at 2 p.m. coupled with an appearance of general weakness. At 4.15 frothing at the mouth occurred, with exceedingly rapid pulse and respiration. The animal was very thin. The dog was etherized at 5.30 p.m., blood withdrawn from the carotid artery and the liver taken for glycogen determination. From 100 grams of the liver, weight 239 grams, not a trace of gly-

⁶ Frank and Isaac: *Arch. f. exp. Path. u. Pharm.*, lxiv, p. 274, 1911.

⁷ Underhill: *this Journal*, x, p. 159, 1911.

cogen⁸ could be obtained. Blood sugar content,⁹ estimated upon 60 grams of blood, was equivalent to 0.03 per cent. Urine collected up to the time of death had no influence upon the plane of polarized light.

Experiment 2. Under morphine-ether anaesthesia the thyroids and parathyroids were removed from a bull bitch of 14.6 kilos. On the following day the dog seemed quiet—water, drunk, was immediately vomited. During the succeeding day the dog was normal in actions. No food was given throughout. The next day the dog was very weak and tremors of the shoulder muscles were observed in the early morning. The animal had lost a great deal of weight. The dog was etherized at noon and blood withdrawn. Glycogen was absent from the liver. Blood sugar, estimated in 23 grams of blood, was present to the extent of 0.035 per cent. Urine was always free from substances having an action upon the plane of polarized light.

Experiment 3. From a pregnant bitch an attempt was made to remove the parathyroids and to leave the thyroids intact. Apparently this was accomplished although the dog gave no evidences of abnormality for a period of twelve days, during which time food was eaten normally and birth was given to five pups, all of which died. From the lack of symptoms it was concluded that some parathyroid tissue had not been removed so under ether anaesthesia alone the remainder of the visible thyroid-parathyroid apparatus was removed. The bitch, now weighing 5.4 kilos, made a rapid recovery and showed no symptoms for forty-eight hours, except that food and water were vomited. At the end of this period tremors were observed, especially of the head, accompanied by chattering of the teeth. The animal, body-weight 5.1 kilos, was etherized and blood collected. Blood sugar estimated on 28 grams amounted to 0.03 per cent dextrose. Glycogen was absent in 100 grams of liver. The urine voided just before death contained a trace of reducing substance. The body temperature was 40.2°C.

Experiment 4. Like Experiment 3 this experiment was planned to study the effect of parathyroid removal. The dog was a bitch

⁸ Pflüger: *Arch. f. d. ges. Physiol.*, cxi, p. 307, 1906.

⁹ Vosburgh and Richards: *Amer. Journ. of Physiol.* ix p. 35 1903

of 13.6 kilos. Inasmuch as only one parathyroid on the right side could be discovered the entire apparatus on the right side was removed, together with both parathyroids from the left side. For a period of a week no symptoms were observed except at intervals a slight twitching of the head muscles. Accordingly the remaining thyroid was removed. For the following two days the peculiar twitchings of the head muscles were noticeable and the dog could not eat owing to the chattering of the teeth. On the next day these symptoms became so pronounced that the dog was etherized and blood drawn. The liver contained no glycogen. Blood sugar content amounted to 0.045 per cent. The body weight at death was 12.8 kilos, the temperature was 39.3°C.

The influence of total parathyroid removal.

Experiment 5. From a bitch of 5.7 kilos and under ether anaesthesia all the thyroid-parathyroid mechanism was removed with the exception of one thyroid. A great thirst developed on the following day but little or no water was retained. On the second day subsequent to the operation tremors were in evidence together with a rapid pulse and respiration. Blood was collected in the usual manner and from 20 grams of it no weighable trace of cuprous oxide could be obtained. Glycogen was also absent from the liver.

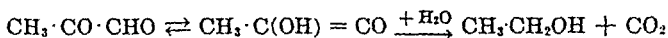
THE FORMATION OF BENZOYL CARBINOL AND OTHER SUBSTANCES FROM PHENYL GLYOXAL BY THE ACTION OF FERMENTING YEAST.

By H. D. DAKIN.

(From the Herter Laboratory, New York.)

(Received for publication, April 20, 1914.)

It has frequently been suggested that methyl glyoxal may play a rôle in alcoholic fermentation of sugar, but hitherto experiments by various workers have failed to show that yeast or zymase has the power of converting methyl glyoxal into alcohol and carbon dioxide.



It appeared of interest to study the effect of actively fermenting yeast upon phenyl glyoxal as the properties of the aromatic substances formed were likely to render them more readily identified than in the case of the simpler compound.

The experiment was carried out in a simple fashion. Fresh washed brewers yeast (300 grams) was added to 10 per cent glucose solution (3000 cc.) and after active fermentation had started a saturated aqueous solution of phenyl glyoxal (10 grams) was dropped in during the course of six hours.

After standing at room temperature over night, the liquid was distilled. The distillate was diluted with water, extracted with a little ether. The ether solution was mixed with water (10 cc.) and the ether removed by distillation. Less than 0.5 gram of oily distillate was obtained which gave the reactions of benzyl alcohol together with a little benzaldehyde. The amount of benzyl alcohol was insufficient for redistillation.

The fermented fluid was evaporated to small bulk *in vacuo* and extracted with ether. The ether extract on treatment with hot water was separated from an oily substance which could not

be purified. The aqueous solution was then exactly neutralized with sodium hydroxide and again extracted with ether. The ether extract on evaporation readily crystallized and gave about 2 grams of practically pure benzoyl carbinol. The substance was purified by recrystallization from boiling petroleum and crystallized in large shining plates melting at 85–86°. The substance gave correct results on analysis:

	Found:	Calculated:
C.....	70.4	70.6
H.....	6.1	5.9

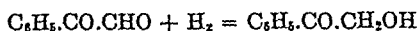
The benzoyl carbinol reduced Fehling's solution in the cold, gave benzaldehyde on boiling with dilute sodium hydroxide and on short treatment with phenyl-hydrazine acetate gave the phenyl hydrazone crystallizing from alcohol in needles melting at 110–112°. On prolonged warming with excess of phenyl hydrazine, osazone formation took place with the production of the diphenyl-hydrazone of phenyl glyoxal, melting after crystallization from alcohol at 152°. The benzoyl carbinol was identical in every respect with the synthetic product.

The neutral aqueous solution from which the benzoyl carbinol had been removed was acidified and extracted with ether. On evaporation there was obtained a crystalline residue weighing about 3 grams containing about 80 per cent of *l*-mandelic acid, together with traces of phenyl glyoxylic acid. On recrystallization from a little water the *l*-mandelic acid was obtained pure, melting at 133° and possessing the full optical rotation. The formation by yeast of *l*-mandelic acid from phenyl glyoxal as the result of the action of glyoxalase has already been described by Dudley and the writer. Phenyl glyoxylic acid was detected in the mother liquor by means of the thiophene reaction and by precipitation with phenyl hydrazine. The amount was very small and could not be satisfactorily purified. No other substances were detected.

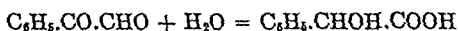
To sum up: the main products of the action of fermenting yeast upon phenyl glyoxal are benzoyl carbinol and l-mandelic acid. Smaller amounts of benzyl alcohol, benzaldehyde and phenyl pyruvic acid were detected.

The formation of a ketonic alcohol such as benzoyl carbinol by biochemical synthesis has not been previously observed. Its

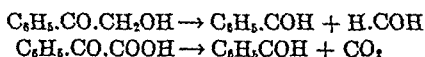
formation clearly involves reduction of the phenyl glyoxal and in this respect resembles the reduction by yeast of aliphatic aldehydes to alcohols observed by Neuberg and others.



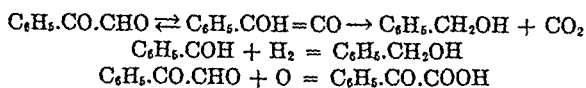
The *l*-mandelic acid was undoubtedly formed by asymmetric hydrolysis by means of enzymes of the type of glyoxalase.



The benzaldehyde is probably derived in part from the benzoyl carbinol which is an unstable substance readily giving benzaldehyde under a variety of conditions or from the action of carboxylase upon phenyl glyoxylic acid.



The benzyl alcohol may be derived directly from the phenyl glyoxal or indirectly from the reduction of benzaldehyde while the phenyl glyoxylic acid would appear to be necessarily derived from the oxidation of phenyl glyoxal.



The behavior of other glyoxals under analogous conditions will be investigated.

THE SUPPRESSION OF GROWTH AND THE CAPACITY TO GROW.¹

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Although the capacity to grow has been the subject of considerable speculation, comparatively little experimental study has been devoted to it. Many of the current statements are scarcely more than dogmatic assumptions based upon hypothesis rather than controlled evidence. In general the dominant impression seems to be that the capacity to grow belongs essentially to the earlier periods of the life cycle; or at any rate it is believed to be more characteristic of that time. Accordingly it would appear that this unique property of living substance is sooner or later lost—at least in so far as it pertains to the higher animal forms.

Quite aside from any consideration of the ultimate cause of growth, the capacity to grow is currently further associated with a youthful character of the cells involved. Age is thereby made a factor of importance in the possibility of growth. An embryonic condition of the cells appears, from this viewpoint, to be most favorable to true growth. In dealing with this function Minot has emphasized his conception of the influence of age by the statement that "the rate of growth depends on the degree of senescence."²

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

² Minot: The Four Laws of Age, *Popular Science Monthly*, lxxi, p. 523, 1907.

From a purely theoretical standpoint it by no means follows that the familiar cessation of growth is due solely to a loss of a capacity to grow incidental to age. It is quite conceivable that natural *inhibitory* factors develop in the course of time. How firmly the idea that the growth-power inevitably declines and is lost with age has become rooted in physiological literature is readily disclosed by a few quotations from well known writers.

Development and the changes involved in growing old are, however, by no means synonymous, so that although in those animals with a fixed size there are always to be found undeveloped cells, yet it is not a correct inference that these cells are also young in the sense that they might still complete their development. It appears, rather, that the capacity for undergoing expansive change is transient, and that those cells which fail to react during the proper growing period of an animal have lost their opportunity forever.³

Wir wissen eigentlich gar nicht, ob die Natur ein absolut gleichmässiges tägliches Wachstum verlangt, oder ob Remissionen zulässig oder gar zweckmässig sind. Nur das steht sicher, dass die Behinderung des Wachstumstriebes, wie dies wirklich vorkommt, nicht während der ganzen Wachstumsperiode andauern darf, da sonst allerdings die Grösse des Individuums dauernd Schaden leidet. Verlorene Körpergrösse in der Jugendzeit kann nach Vollendung der Wachstumsperiode nimmermehr abgeglichen werden.⁴

Again we read:

Das schliessliche Versagen des Wachstums hängt auch natürlich nicht mit einer ungenügenden Ausbildung der Resorption der Nahrung zusammen; ein eben ausgewachsenes Individuum kann das Mehrfache an Nahrung resorbieren von dem, was an Unterhalt nötig ist, den Wachstumstrieb wird man nimmer wecken. . . . Das Tier hört bei geringer Ernährung auf, überhaupt zu wachsen, vollzieht aber seine sonstige biologische Entwicklungsperiode. Obschon also das Wachstum künstlich gehemmt werden kann und somit latent bleibt, verliert sich trotzdem der Wachstumstrieb. Die Wachstumsfähigkeit kann in ihrer Begrenzung nicht von einer nur im Wachstum selbst entstehenden Schädigung oder einem mit der Zellmasse und Zellbildung unmittelbar zusammenhängenden Vorgang gebunden sein, weil sie auch da schliesslich schwindet, wo sie überhaupt nicht in Anspruch genommen worden ist.⁵

³ Donaldson, H. H.: *The Growth of the Brain*, New York, 1903, p. 37.

⁴ Rubner, M.: *Das Problem der Lebensdauer und seine Beziehungen zu Wachstum und Ernährung*, München und Berlin, 1908, p. 82; also Ernährungsvorgänge beim Wachstum des Kindes, *Arch. f. Hygiene*, lxxvi, p. 82, 1908.

⁵ Rubner, M.: *Kraft und Stoff im Haushalte der Natur*, Leipzig, 1900, pp. 116-17.

Further:

Alle Mittel, alle Versuche unsere alternden Zellen mit verjüngender Kraft zu versehen, sind eitel; nichts kann den Verfall hemmen. Nur die Befruchtung vermöchte neues Leben zu schaffen. Diese Hilfe ist uns aber versagt, sie gilt nur den Fortpflanzungszellen, der neuen Generation, der Zukunft.⁶

In der Regel ist, bei den verschiedenen Tieren verschieden stark, der Wachstumstrieb in der Jugend am stärksten, nimmt allmählich ab und verliert sich im Alter. Die Natur des Wachstumstriebes ist dunkel. Sie ist eine Funktion der Zellen, im besonderen der jugendlichen Zellen. Welche Faktoren diesen Zelltrieb regulieren, wissen wir nicht, vor allem nicht, warum er allmählich aufhört. Ob hier die Zeitdauer seiner Wirksamkeit, ob die erreichte Grösse des Individuums den Ausschlag für das Abklingen des Wachstumstriebes gibt, ist bis jetzt nicht entschieden.⁷

A somewhat different view is expressed by Friedenthal:

Inwieweit eine Zelle wachstumsfähig ist, hängt in hohem Masse von der Zahl der bereits abgelaufenen Zellteilungen ab, welche zur Bildung dieser Zelle von der befruchteten Eizelle her führte. Wir dürfen jeder befruchteten Eizelle die Möglichkeit der Erzeugung einer artmässig verschiedenen Zahl von Zellteilungen zuschreiben, nach deren Ablauf die Regenerationskraft des Organismus erschöpft ist, so dass der Tod als notwendige Folge der nun irreparabel gewordenen Schädigungen der Arbeitsmaschine des Lebewesens anzusehen ist. Je mehr wir imstande sein werden, den Rhythmus der Zellteilungen beim Menschen zu verlangsamen, um so höher werden wir die Lebensdauer und die Gesamtleistung des Menschen zu steigern vermögen.

The facts gleaned from the study of the regeneration of parts, as it is exemplified in the lower forms of animals, suggest a further alternative. It may be that the capacity to grow is limited by the exercise of this capacity rather than by age, as such. The problem cannot be solved by *a priori* considerations. It is amenable to experiment.

Before presenting our own experience in this field we must point out a distinction between growth, as we understand it, and the related process of repair or regeneration of depleted parts and

⁶ Rubner, M.: *ibid.*, p. 180.

⁷ Aron, H.: Wachstum und Ernährung, *Biochem. Zeitschr.*, xxx, p. 207, 1910.

⁸ Friedenthal, H.: Das Wachstum des Körpergewichts des Menschen und anderer Säugetiere in verschiedenen Lebensaltern, *Zeitschr. f. allgemeine Physiol.*, ix, p. 487, 1909; also *Arbeiten aus dem Gebiet der exp. Physiol.*, ii, p. 51, 1911.

organs. There are indications already available that the chemical or metabolic processes of repair are by no means identical with growth. They may not involve the destruction and resynthesis of an entire protein molecule or of the entire protoplasmic cell structure. Furthermore it is a matter of common experience that repair or recuperation can take place at all ages and even after the completion of normal growth; though it may be desirable to consider the ability to recuperate with reference to age. There is a common belief that children recover weight more promptly than adults. We have repeatedly observed that animals which have declined markedly in body weight regain the weight normal for their age when returned to a proper diet and health conditions, at a rate far surpassing that observed in the original growth of individuals through the same range of body weight. The process of restoration is accomplished with wonderful rapidity.⁹ A striking illustrative instance of this is depicted in the Appendix (Chart I). Nevertheless it must be admitted as possible, though scarcely probable, that restored individuals may not be in all respects normal. Qualitative changes not appreciated by the cursory examination may have become permanently engrafted.¹⁰

That growth can be retarded or even suppressed for brief periods *at an early age* without loss of the capacity to complete it is known. Hatai, for example, has found that "so far as the weight of the body and central nervous system are concerned, the effect of a twenty-one day period of partial starvation on albino rats thirty days old is eventually completely compensated."¹¹

To Hans Aron we owe elaborate experiments on the influence of the suppression of growth during the early period of life upon the capacity of animals to complete their normal development. He accomplished this retardation by underfeeding. The earlier experi-

⁹ Cf. Morgulis, S.: Studies of Inanition in its Bearing upon the Problem of Growth, *Arch. f. Entwicklungsmechanik der Organismen*, xxxii, p. 169, 1911.

¹⁰ Cf. Donaldson, H. H.: President's Address before the Philadelphia Neurological Society, *Journ. of Nervous and Mental Disease*, xxxviii, p. 257, 1911.

¹¹ Hatai, S.: Effect of Partial Starvation Followed by a Return to Normal Diet, on the Growth of the Body and Central Nervous System of Albino Rats, *Amer. Journ. of Physiol.*, xviii, p. 309, 1907.

ments,¹² carried out on dogs, demonstrated that despite the considerable inhibition of growth which was not, however, extended throughout the entire period of adolescence, the animals still retained a capacity to grow vigorously. This is in contrast to the added observation that if the restriction in the diet and the inhibition of growth has been extended throughout the entire customary period of growth, the capacity to grow is apparently lost. For example, of two dogs approximately three months in age, one was nourished normally and the other kept at approximately constant weight of about 2400 grams over a period of 310 days. At the end of this period the normal animal had reached a weight of 6800 grams. The stunted individual was now fed abundantly, whereupon it added considerable fat to its body. Nevertheless at the end of 500 days it had showed little increase in body length despite the increment of body weight, and it remained considerably behind its normal companion in size. More recent experiments by Aron¹³ on rats along the same lines have shown that at an early age the growth of these animals may be retarded by under-feeding for considerable periods of time and yet a capacity for subsequent vigorous growth be retained. This investigator remarks, however: "Allerdings scheinen die Tiere weder in Gewicht noch in Grösse ihre normalen Brudertiere völlig zu erreichen und das Versäumte nicht ganz nachholen zu können." Taken in their entirety, therefore, these experiments still leave the question as to the relation of age to the capacity to resume growth unsettled.

Although Rubner¹⁴ has only lately maintained that the capacity to grow finally disappears even when it is not called into use, Aron¹⁵ has pointed out in a more recent review of the subject of growth that the experimental evidence is contrary to this assump-

¹² Aron, H.: Wachstum und Ernährung, *Biochem. Zeitschr.*, xxx, p. 207, 1910; Nutrition and Growth, I, *Philippine Journal of Science*, vi, Sec. B, p. 1, 1911.

¹³ Aron, H.: Weitere Untersuchungen über die Beeinflussung des Wachstums durch die Ernährung, *Verhandlung der 29 Versammlung der Gesellsch. f. Kinderheilk. in der Abteilung f. Kinderheilk. der 84 Versamml. d. Gesellsch. deutsch. Naturforsch. u. Aerzte in Münster*, 1912, 103.

¹⁴ Rubner, M.: *Kraft und Stoff im Haushalte der Natur*, Leipzig, 1909, pp. 116-117.

¹⁵ Aron, H.: Biochemie des Wachstums des Menschen und der höheren Tiere, *Handbuch der Biochemie*, Ergänzungsband, Jena, 1913, p. 70.

tion.¹⁶ He refers in particular to our published observations¹⁷ in which the capacity to grow was shown to be maintained at the age of 314 days after an inhibition of 276 days. Data are quoted to support the belief that the final adult size of the body may be markedly affected by *very prolonged* undernutrition during the period of adolescence. In discussing the growth of children Boas¹⁸ has noted that although retardation of early growth is made up by abnormally rapid development at a later period, an unduly prolonged retardation cannot be entirely compensated for; accordingly excessive early inhibition of growth may be detrimental to the individual. Although the growth of the body in recuperation is very rapid in children whose development has been temporarily disturbed by disease or other unfavorable conditions, what will happen to them in the latter portions of the span of life has yet to be determined in order to answer the question whether the partial starvation in early life has any influence either on longevity or on the onset of old age.¹⁹ Aron points out that most of the scanty experimental evidence heretofore collected on this topic pertains to subjects which have been stunted in their growth by underfeeding; and he raises the question as to whether the conclusions drawn from such a mode of inhibition apply equally well to other conditions of retarded growth.

Die Wachstumsfähigkeit, und die Intensität des Wachstumstriebes kann nach den Erfahrungen der beschriebenen Versuche nicht mehr vornehmlich als eine Funktion des Alters, d. h. der nach dem Befruchtungsvorgang verstrichenen Zeitspanne oder der im Körper abgelaufenen Zahl der Zellteilungen betrachtet werden, auch nicht von der umgesetzten Kalorienzahl abhängig sein. Massgebender als das Alter scheint die endgültige Grösse des Individuums zu sein, wie die folgende Beobachtung dartun soll: Weibliche Ratten erreichen ausgewachsen nur ein erheblich geringeres Gewicht als männliche Geschwistertiere; ihre Wachstumskurve verflacht sich früher

¹⁶ Schulz, P.: Wachstum und osmotischen Druck bei jungen Hunden, *Zeitschr. f. Kinderheilk.*, iii, p. 251-56, 1911.

¹⁷ Osborne and Mendel: Beobachtungen über Wachstum bei Fütterungsversuchen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-70, 1912; The Role of Gliadin in Nutrition, *this Journal*, xii, pp. 473-510, 1912.

¹⁸ Boas, F.: The Growth of Children, *Science*, Dec. 13, 1912, p. 815.

¹⁹ Cf. Hatai, S.: Effect of Partial Starvation followed by a Return to Normal Diet, on the Growth of the Body and Central Nervous System of Albino Rats, *Amer. Journ. of Physiol.*, xviii, p. 315, 1907.

und ihr Wachstum stockt vor dem der Brudertiere gänzlich. Hemmt man nun eine junge männliche Ratte im Wachstum so lange bis ein Schwestertier ausgewachsen ist und das gehemmte Brudertier erheblich an Gewicht übertrifft, und füttert jetzt erst das gehemmte Brudertier auf, so erreicht endgültig doch das männliche Tier trotz der Wachstumsheftung noch ein beträchtlich höheres Gewicht als das Schwestertier, ein Ausdruck der überragenden Wirkung der kongenitalen Anlage (des Wachstumstriebes) auf den Ablauf der Wachstumsvorgänge gegenüber sekundären Momenten.²⁰

EXPERIMENTAL PART.

In testing the capacity of albino rats to continue their growth after more or less prolonged inhibition of this function, as manifested by an approximately stationary body weight, we have attempted the suppression of growth by a variety of procedures.²¹ Instead of the method of underfeeding with rations of suitable qualitative make-up we have induced effective stunting by a variety of other ways such as: the use of diets containing an adequate protein, but with the inorganic salts supplied in the form of a mixture of pure chemicals, as used by Röhmann, together with sucrose and starch as the carbohydrate component (Chart IV); by restricting the protein content of the dietary below the minimum required for growth; by furnishing, as the exclusive source of nitrogenous intake, proteins which lack some amino-acid group indispensable for growth (Charts III and IV).²² In all of these experiments care has been exercised to prevent any prolonged decline of body weight on the dietary régimes employed. Since some of them are unsuited even for brief maintenance alone, it has become necessary to resuscitate the rats at intervals, by administration of some perfect nutrient like our milk-food mixtures. As soon as the animals were brought back in this way to their previous maximal weight, or even somewhat above it, the inhibitory diet was renewed (see Charts IV and V).

²⁰ Aron, H.: *Biochemie des Wachstums des Menschen und der höheren Tiere, Handbuch der Biochemie, Ergänzungsband*, Jena, 1913, p. 71.

²¹ Cf. Osborne and Mendel: Beobachtungen über Wachstums bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-70, 1912.

²² Cf. Osborne and Mendel: Amino-acids in Nutrition and Growth, this *Journal*, xvii, p. 325, 1914.

The results of some of these trials are presented graphically in appended charts. In interpreting them it is necessary to bear in mind the usual duration of the normal period of growth. This is extremely variable; but it rarely exceeds 335 days. Rigid limits in respect to duration of growth and the maximum size attained cannot be given. The males invariably grow to a larger size than the females. Only four out of 110 rats, all females, increased in weight after one year of age; out of 38 males only four increased in weight after 300 days. Beyond the age noted above, increase in body weight is extremely slow, if it occurs at all; and probably it represents depositions of reserve materials, like fat, rather than growth in the sense of developmental changes.

In some of the stunted animals the momentary body weight on the day in which an effective diet for growth was resumed is below that which they had reached at some previous date. An increment in weight up to the figure for the previous maximum cannot in fairness be considered as new growth, inasmuch as it may represent something merely analogous to repair. Therefore we have indicated by a "basal line" in some of the curves the point at which the weight began to increase above the previous maximum, and considered this as the beginning of new growth.

In Rat 531♀, Chart II, the capacity to grow was not lost at the age of 532 days, or after more than half the life-span of most of the rats kept under our conditions of housing and caging.²³ This animal, it must be noted, was already well grown to a size of 170 grams before the stunting period on corn gluten food²⁴ checked further increase in weight. Rat 569♀, Chart III, similarly stunted, at 160 grams of body weight, showed a vigorous growth response at the age of 480 days. Chart IV exhibits the resumption of growth at 418 days of age after a period of very prolonged suppression of growth by the method of alternate feeding of imperfect and perfect nutrient mixtures (indicated by the straight and interrupted lines respectively). A similar experience is indicated by Chart V in which the inhibition of growth and nutritive

²³ Slonaker (*Journ. of Animal Behavior*, ii, p. 20-42, 1912) has kept albino rats alive for more than one thousand days in the laboratory.

²⁴ Cf. Osborne and Mendel: Nutritive Properties of Proteins of the Maize Kernel, this *Journal*, xviii, p. 1, 1914.

decline was due to the interrupted use of gelatin as the exclusive source of nitrogen in the dietary.

The illustrative experimental results here reviewed give no support to the widespread view that the capacity to grow—*Wachstumstrieb*, *Wachstumsfähigkeit*, or growth impulse, as it has been variously termed—is lost with age, independently of whether it has or has not functioned during the period usually associated with increase in size. The difficulties of continuing the trials of the sort here exemplified over periods of years, rather than months, are great; so that we are not in a position to say that the growth impulse will never become exhausted in ungrown animals in the course of time. But it appears as if the capacity to grow is only lost by the exercise of this fundamental property of animal organisms. .

These conclusions have not been attained by a single procedure; for our animals were prevented from growing by a variety of methods. Whether the span of life can be prolonged by delaying the completion of growth has not yet been ascertained under conditions that preclude disease or other anomalies of nutrition. Like the questions relating to possible changes in morphological or chemical make-up of the tissues incident to suppression of growth, this one is open to experimental study.

APPENDIX.

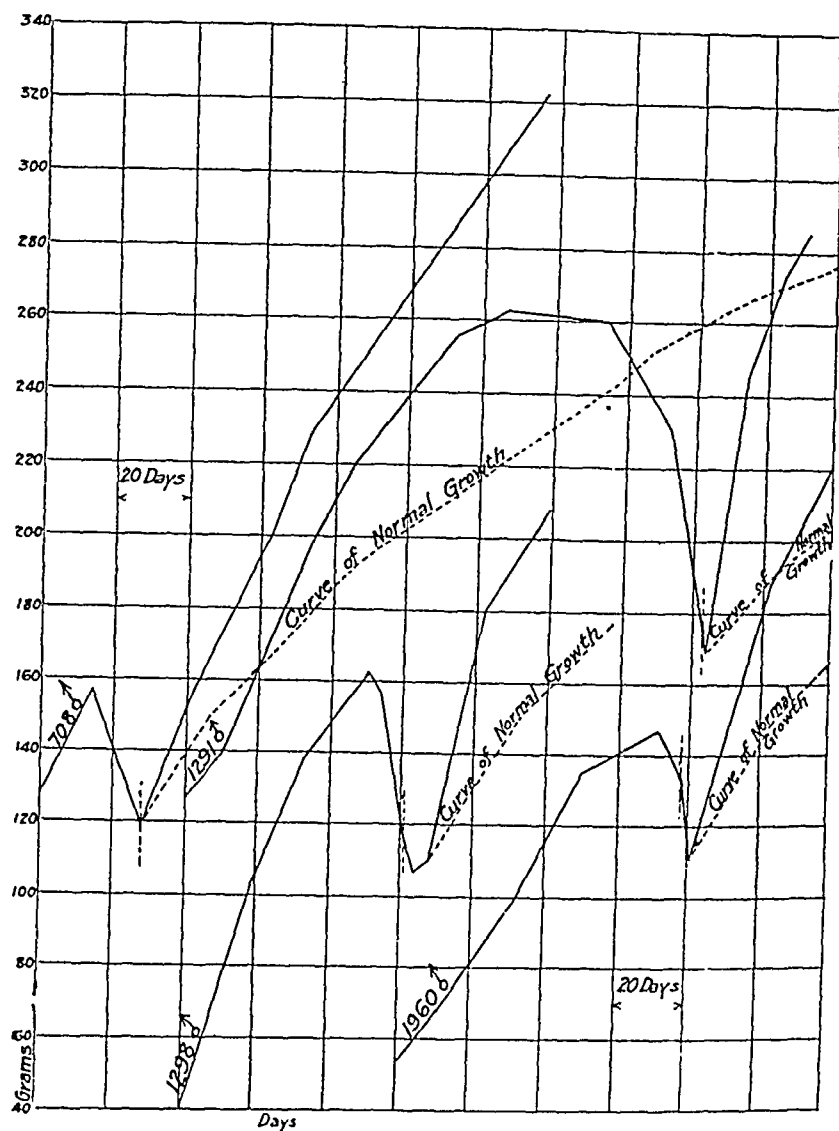


CHART I, showing curves of repair after a considerable fall in body weight due to feeding with a defective diet. Note that lost weight is regained far more rapidly than during normal growth at the same size.

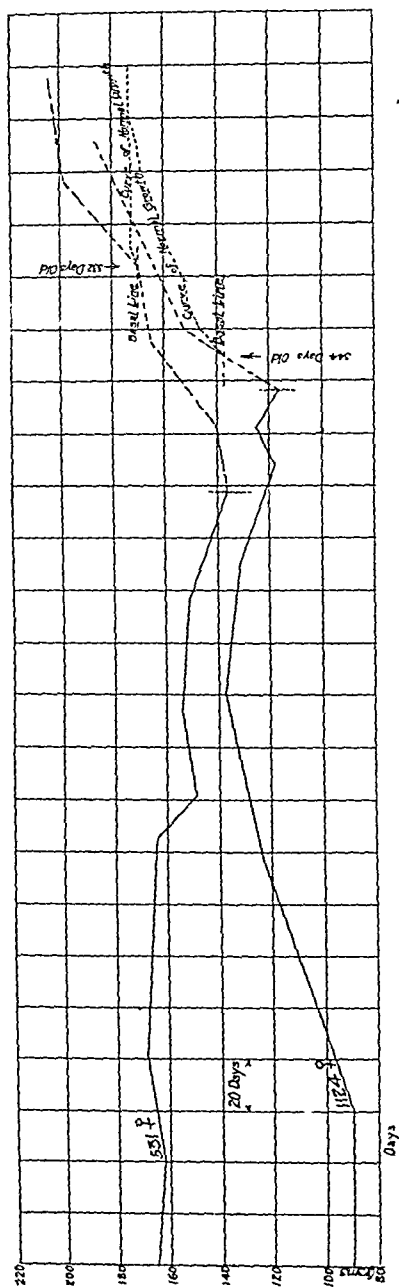


CHART II, showing capacity to grow at 532 days of age and 170 grams of body weight; and 344 days and 137 grams respectively.

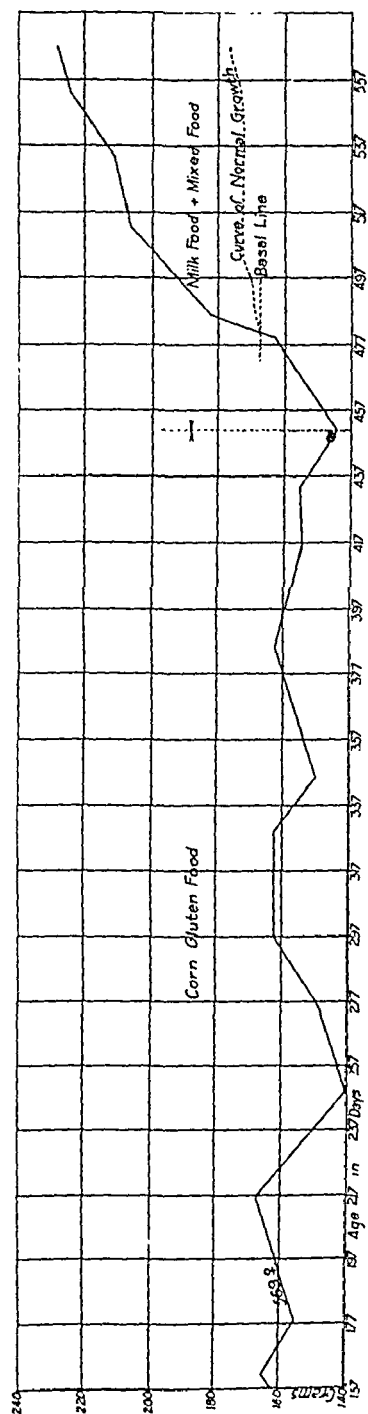


CHART III, showing capacity to grow at 480 days of age and 167 grams of body weight.

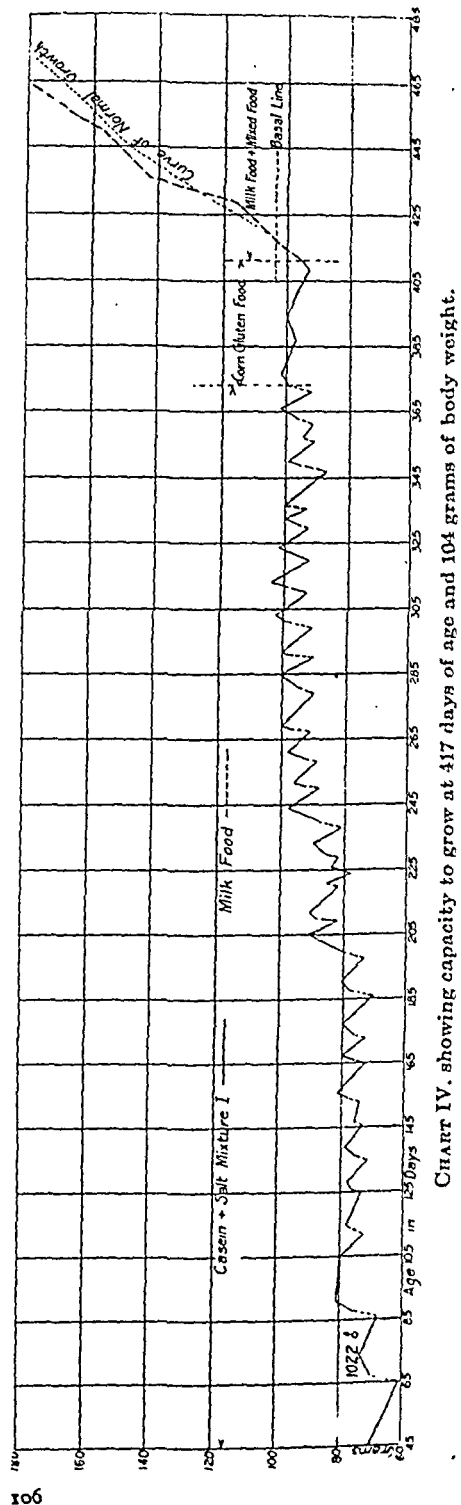
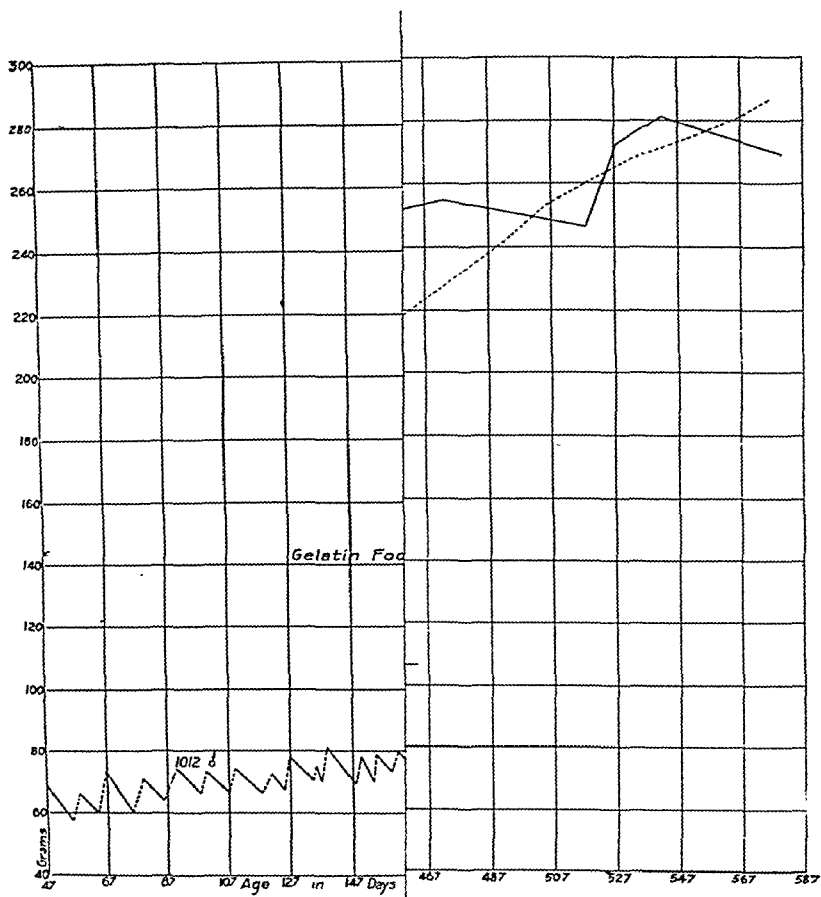


CHART IV, showing capacity to grow at 417 days of age and 104 grams of body weight.



1012 d.

THE METABOLISM OF ENDOGENOUS AND EXOGENOUS PURINES IN THE MONKEY.

THIRD PAPER.

THE PURINES OF MONKEY URINE.

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(Received for publication, May 1, 1914.)

While in many, possibly most, mammalian species the urinary output of purine bases is smaller than that of uric acid, there are some in which this relation is, either regularly or occasionally, reversed. A preponderance of bases has been observed, for example, in several ungulates, such as the pig,¹ the horse,² the sheep,³ and the goat;⁴ and in the earlier papers of this series⁵ it has been shown to be characteristic also of the monkey, or at least of one species of monkey, *Cercopithecus callitrichus*. It is not impossible that the comparative study of purine excretion in a wide range of forms would disclose many other instances of the same peculiarity.

The condition seems to imply, in those animals that exhibit it, a relative retardation of the processes that bring about the transformation of amino-purines into uric acid. For the more precise determination of the point or points in the chain of enzymatic reactions, where such retardation is effective, there are two available modes of inquiry. One is the direct study of the purine enzymes demonstrable in the tissues, the other the analytical determination of the proportions in which the different amino- and

¹ Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xlviii, p. 140, 1906; Schittenhelm: *ibid.*, lxvi, p. 53, 1910; Mendel and Lyman: this *Journal*, viii, p. 115, 1910.

² Schittenhelm and Bendix: *loc. cit.* The findings of these authors in relation to horse urine have, however, not been confirmed in this laboratory.

³ Hunter: *Quart. Journ. of Physiol.*, viii, p. 13, 1914.

⁴ Hunter and Givens: this *Journal*, xvii, Proceedings, p. xxiii, 1914.

⁵ Hunter and Givens: this *Journal*, xiii, p. 371, 1912, and xvii, p. 37, 1914.

oxypurines appear in the urine. Neither method is capable by itself of giving all the information required. That obtained by the first is mainly qualitative in character; we have at present no means of estimating the relative amounts or actual efficiency of the enzymes which any tissue or organ may be shown to contain. The deficiencies of the second method are quite as serious; on the one hand there are often several routes by which a urinary purine may have come into existence, and on the other the procedure for analyzing a mixture of purine bases is far from being strictly quantitative. Whether for any of the animals mentioned it will be possible, by making the most of both lines of attack, to fully elucidate the point in question, remains to be seen; certainly nothing short of this is likely to accomplish the object.

The purine enzymes of monkey tissues have formed the subject of a report by Wells,⁶ who found adenase and guanase to be widely distributed, while xantho-oxidase and uricase were detected in the liver alone. These statements are based almost entirely upon experiments with *Macacus rhesus*. In the organs of *Cebus apella*, of which one specimen was examined, the two deaminizing enzymes could be demonstrated, although upon adenine the action of this monkey's liver was hardly as striking as in the case of *Macacus*. As to the purine enzymes of *Cercopithecus* no information whatever exists, and any assumption in respect of them must in the meantime rest upon the rather insecure foundation of analogy with the other monkeys mentioned.

The present communication records an attempt to throw light upon the problem, as far as *Cercopithecus* is concerned, by a determination of the approximate composition of the purine mixture excreted. During the long period in which the experimental animal was under observation in this laboratory the residues of urine not required for the routine determinations were systematically, as they accumulated to a convenient bulk, treated by the Krüger-Schmidt process for the precipitation of purines.⁷ The precipitates were decomposed by hydrogen sulphide in the presence of hydrochloric acid, and the filtrate from copper sulphide was evaporated to a comparatively small bulk. From this the purines were a second time precipitated; the second precipitate

⁶ Wells: this *Journal*, vii, p. 171, 1910.

⁷ Care was taken to exclude urine that might contain exogenous purines.

was treated like the first, and the final product was carefully evaporated to dryness. In this way there were at length collected several grams of crude purine hydrochlorides, containing, however, as afterwards appeared, a very considerable amount of inorganic material. To this mixture there was applied a scheme of separation based upon the methods of Krüger and Salomon⁸ and Krüger and Schittenhelm.⁹

The whole was first digested twelve hours at 40° with about 50 cc. of water. The residue (A) was then separated by filtration under suction from the solution (B).

(A) The residue gave the xanthine test and the Folin-Macallum color reaction with phosphotungstic acid. It was dissolved in about 100 cc. of warm 2 per cent NaOH, and, after cooling, filtered from a considerable inorganic residue, which contained much calcium and some iron. The filtrate was acidified with acetic acid, whereupon a bulky gelatinous precipitate (of xanthine) fell out, and then treated warm with so much hydrochloric acid that the precipitate, with the exception of a small heavy crystalline remainder, went again into solution. After cooling to 0° the crystalline deposit, considerably increased in bulk, was collected on a filter, washed, and dried. It weighed 0.35 gram. Its crystalline form, and the fact that it gave the murexide test and an intense blue reaction with phosphotungstic acid indicated that it consisted mainly of *uric acid*. For analysis it was purified by the method of Horbaczewski.

NITROGEN (Kjeldahl): 0.1201 gram required 28.4 cc. $\frac{N}{10}$ acid.

	Calculated for $C_5H_4N_4O_6$	Found:
N.....	33.33	33.13

The yield of purified uric acid was 0.32 gram.

The filtrate from uric acid gave no precipitate with ammonia, and contained therefore no guanine. It was rendered alkaline with NaOH, and acidified with acetic acid. A considerable precipitate separated at once. After a day this was collected, purified by solution in NaOH and reprecipitation by acetic acid, washed, dried at 110°, and analyzed.

⁸ Krüger and Salomon: *Zeitschr. f. physiol. Chem.*, xxvi, p. 350, 1898.

⁹ Krüger and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xxxv, p. 153, 1902.

NITROGEN (Kjeldahl): 0.1222 gram required 31.84 cc. $\frac{N}{16}$ acid.

	Calculated for $C_5H_4N_4O_2$:	Found:
N.....	36.85	36.50

The substance was therefore *xanthine*; the amount here obtained was 0.85 gram.

The filtrate from this first crop of xanthine was treated with ammonia and silver nitrate. From the silver precipitate there was isolated in the usual way 0.24 gram of free purine. This was dissolved in 3 cc. of 3 per cent NaOH and poured into 50 per cent HNO_3 . The nitrate which separated on standing had the crystalline form typical of xanthine nitrate. There was obtained from it, by solution in alkali and precipitation with acetic acid, 0.1 gram of practically pure base.

NITROGEN (Kjeldahl): 0.0877 gram required 22.8 cc. $\frac{N}{16}$ acid.

	Calculated for $C_5H_4N_4O_2$:	Found:
N.....	36.85	36.4

By working up the filtrates from xanthine nitrate and from the subsequently isolated base, there was obtained a further small crop of material, of which the nitrate resembled microscopically that of xanthine. The yield, however, was too small for analysis. The total amount of xanthine positively identified was therefore 0.95 gram. Purines other than uric acid and xanthine must have been present in the residue A in very small quantities, if at all.

(B) The solution B was made up to a volume of 100 cc., and treated, for the separation of any possible guanine, with 20 cc. of 10 per cent ammonia. There was an immediate flocculent precipitate, which, however, turned out to be entirely inorganic, consisting for the most part apparently of ferric hydroxide. Guanine was evidently absent. The filtered solution was accordingly evaporated, and the purines converted once more into hydrochlorides. These were dissolved in about 100 cc. of water, filtered from a considerable quantity of calcium sulphate, which separated at this point in crystalline form, and treated with sodium picrate. Only a small flocculent precipitate was produced. This was filtered off and washed with a little cold water. From the filtrate the purines were precipitated as copper compounds. The solution

obtained by the decomposition of the latter was neutralized and made up to 200 cc. Of this 1 cc. was utilized for the determination of nitrogen according to the Folin-Farmer micro-method.¹⁰ It was found that the nitrogen present amounted to 0.174 gram, corresponding to 0.42 gram of hypoxanthine. On the basis of this determination there was added 0.8 gram of picric acid, which was brought into solution by heat. On concentrating and cooling there was obtained first a small quantity of an amorphous flocculent precipitate. This was collected by filtration, and added to the earlier precipitate yielded by sodium picrate. The filtrate was further concentrated, when it deposited typical crystals of hypoxanthine picrate. The yield was 0.97 gram, equivalent to 0.36 gram of *hypoxanthine*. The filtrate from this product gave hardly any precipitate with ammoniacal silver nitrate.

The hypoxanthine picrate was dissolved in dilute nitric acid, the solution freed from picric acid by extraction with toluene, and evaporated to a small bulk. Hypoxanthine nitrate separated in characteristic whetstone crystals to the amount of 0.464 gram, corresponding to 0.29 gram hypoxanthine. From this there was obtained in the usual way (solution, treatment with ammonia, and evaporation) 0.26 gram of the free base. Identification was completed by analysis of the latter.

NITROGEN (Kjeldahl): 0.1191 gram required 34.94 cc. $\frac{N}{10}$ acid.

	Calculated for $C_5H_4N_4O_2$	Found:
N.....	41.17	41.1

The filtrates from hypoxanthine nitrate and hypoxanthine were combined and precipitated with silver nitrate. The precipitate yielded upon decomposition 0.08 gram of a purine, the nitrate of which consisted entirely of the whetstones and barrels typical for hypoxanthine nitrate. It appears therefore justifiable to calculate the actual amount of hypoxanthine present from the weight of the crystalline picrate.

The amorphous precipitates obtained first with sodium picrate, and subsequently with picric acid, must have contained any adenine that was present. The combined material, which was very small in amount, could not in any manner be brought to crystallize. It

¹⁰ Folin and Farmer: this *Journal*, xi, p. 493, 1912.

was therefore dissolved in water, and precipitated with silver nitrate and ammonia. The precipitate was decomposed with hydrogen sulphide, filtered, boiled with charcoal, and evaporated to dryness. The quantity of solid material was extremely minute. Its solution gave no precipitate either with picric acid or with gold chloride. It appears therefore exceedingly improbable that any adenine whatever was present.

The results of the analysis are summarized below.

	gram
Guanine.....	0
Adenine.....	0?
Hypoxanthine.....	0.36
Xanthine.....	0.95
Uric acid..	0.32

A study of the purine bases of urine, such as is here recorded, has been seldom attempted. The classical investigation of Krüger and Salomon¹¹ upon human urine, and the reports of Schittenhelm and Bendix and of Schittenhelm¹² alone upon the purine excretion of the pig, appear indeed to be the only previous examples. The numerical data furnished by these authors are compared with those obtained for the monkey in the accompanying table, in which also, to make the relations clearer, the distribution of nitrogen among individual bases has been calculated for each of the animals concerned. Methyl-purines, as not involved in nuclein metabolism, have been omitted from consideration.

	GRAMS SUBSTANCE			DISTRIBUTION OF NITROGEN		
	Monkey	Man	Pig	Monkey	Man	Pig
Guanine.....	0	0	0	0	0	0
Adenine.....	0	3.54	0.023	0	20	7
Hypoxanthine.....	0.36	8.50	0.250	30	39	58
Xanthine.....	0.95	10.11	0.175	70	41	36

It will be seen that in all three cases there is a preponderance of oxypurines over aminopurines, the latter being entirely absent from monkey urine and in the others represented by adenine

¹¹ Krüger and Salomon: *loc. cit.*

¹² Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xlviii, p. 140, 1906; Schittenhelm: *ibid.*, lxvi, p. 53, 1910.

alone. The relative proportions of the oxypurines appear to vary; in man xanthine and hypoxanthine account for nearly equal fractions of nitrogen, while in the pig hypoxanthine, in the monkey xanthine, decidedly preponderates. Upon figures obtained by methods so imperfectly quantitative as those employed it is of course impossible to base any very positive deductions. The most that can be said is that the differences noted are suggestive, and may be significant. It would hardly be surprising if the specific differences revealed in the distribution of purine enzymes were apparent also in the composition of the purine mixture excreted by the kidney.

So far as the results with the monkey are concerned, it may be noted in the first place that the actual isolation and identification of uric acid confirms the conclusion, hitherto based solely on the Folin-Macallum color reaction, that that substance is a regular product of endogenous metabolism; the yield too, in relation to the bases, agrees fairly well with that which the colorimetric determinations would have led one to expect. It is seen, next, that the preponderance of bases over uric acid is due chiefly to the remarkably high proportion of xanthine. This is a circumstance which points directly to a deficiency of xantho-oxidase, a deficiency already rendered probable, not only by the experiments of Wells already cited, but also by the large proportion (30 per cent) of unchanged xanthine which is excreted after subcutaneous injection.¹³ Conversely the absence of aminopurines might on first thought be correlated with the extensive distribution assigned to guanase and adenase in monkey organs. As far as guanine is concerned the correlation would probably be justified, since it is confirmed by the fate of injected guanine, little of which (certainly less than 28 per cent) escapes catabolism.¹⁴ With adenine the case is not so clear; here the experiment of parenteral introduction did not at all suggest that *Cercopithecus* has a notable capacity for deaminizing adenine. If that experiment is to be reconciled with those upon tissue enzymes, it is only by assuming that *Cercopithecus* resembles less *Macacus* than that other species of monkey (*Cebus apella*) in which adenase appeared to be not particularly abundant. To account then for the absence of ade-

¹³ See Hunter and Givens: this *Journal*, xvii, p. 37, 1914.

¹⁴ *Ibid.*

nine from the urine, we should have to suppose further that in *Cercopithecus* the adenine radicle of nucleic acid is normally transformed to hypoxanthine without the intermediary appearance of free adenine at all. In view of the work of Jones¹⁵ upon nucleoside-deaminases such a supposition has about it nothing improbable.

These of course are mere speculations. The only one of the foregoing suggestions in which one may feel a reasonable degree of confidence is that of the relative deficiency of xantho-oxidase. Even here there are grounds for dubiety. The kind of evidence which supports that suggestion is partly paralleled in relation to uric acid. Uricase, like xantho-oxidase, has been demonstrated in the liver alone; and injected uric acid reappears unchanged in even greater proportion than xanthine.¹⁶ Yet the idea of a lack of uricase would seem to be altogether negatived by the monkey's uricolytic index of 90.¹⁷ It is clear that much remains to be done before all the isolated facts connected with nuclein metabolism are reconciled, and that, as already insisted on, no single method of research is likely to lead to complete comprehension of the process.

¹⁵ See Jones: this *Journal*, ix, p. 169, 1911; Amberg and Jones: *Zeitschr. f. physiol. Chem.*, lxxiii, p. 407, 1911.

¹⁶ Hunter and Givens: *loc. cit.*, 1914.

¹⁷ *Ibid.*

THE FORMATION OF GLUCOSE FROM CITRIC ACID IN DIABETES MELLITUS AND PHLORHIZIN GLYCOSURIA.¹

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Although sodium citrate is extensively used in the treatment of diabetic acidosis, no attempt seems to have been made to ascertain whether or not citric acid might itself give rise to glucose. In the course of their work on the effect of various substances on diabetic acidosis, Baer and Blum² administered sodium citrate to a phlorhizinized dog. They observed no particular effect on the excretion of acetone and β -hydroxybutyric acid and did not repeat the experiment. There was, however, a slight rise in the excretion of glucose, the significance of which seems to have escaped them. In his monograph on phlorhizin glycosuria, Lusk³ calculated the ratio of glucose to nitrogen in the urine in this experiment and showed that this was increased considerably on the administration of the citrate. Lusk declared that this probably indicated a formation of glucose from citric acid.

A little more than a year ago, a patient under observation exhibited a G:N ratio of about 3.65, the maximal ratio in phlorhizinized dogs. In passing, it may be mentioned that this patient lived for eight or nine months after this ratio was discovered. Apparently, this patient was a very suitable one for the study of the effect of sodium citrate upon acidosis and glucose excretion. The first experiment gave very striking results. The

¹ Some of the experiments here reported were performed at the Montefiore Home, New York.

² Baer and Blum: *Arch. f. exp. Path. u. Pharm.*, lxx, p. 1, 1911.

³ Lusk: *Ergeb. d. Physiol.*, xii, p. 315, 1912.

output of glucose was increased by an amount corresponding almost exactly to the amount of sodium citrate administered, on the assumption that all six carbon atoms of the citric acid were converted into glucose. The excretion of acetone and β -hydroxybutyric acid was considerably increased, probably as a result of the liberation of a large amount of alkali from the sodium citrate. The experiment was repeated twice. One was apparently negative, no "extra glucose" being eliminated on the days of citrate administration. However, the patient gained over three kilos in weight and the experimental period was succeeded by one of high G:N ratios. The amount of "extra glucose" eliminated in this period was a little less than what might have been formed from the citric acid. It is possible that the glucose was formed and not excreted promptly but retained within the organism. In the third experiment, there was an increase in the G:N ratio after taking sodium citrate but this was not as marked as in the first experiment.

In view of the doubt that always attaches to the results obtained in experiments with patients suffering from diabetes, it was thought advisable to test the action of citric acid upon phlorhizinized dogs. The earlier experiments, in which the sodium citrate was given by mouth, were not satisfactory. As a rule, the G:N ratio was increased slightly but not sufficiently to allow of definite conclusions. Subcutaneous administration was then employed and marked increases in the excretion of glucose and in the G:N ratio were obtained in almost every instance. Unfortunately, sodium citrate is quite toxic and if too large an amount is given, the dog dies within twelve hours. Only experiments in which the animal lived at least forty-eight hours after the administration of the citrate have been considered. The amount of "extra glucose" was, as a rule, very nearly what was to be expected if the six carbon atoms of citric acid had been converted into glucose. In one experiment (table IV, first experiment), the amount of "extra glucose" appears to be greater than could be derived from the citric acid. This animal was rather large and exhibited high G:N ratios in the foreperiod. It seems possible that a small amount of glycogen had been retained and now appeared in the urine as glucose.

The excretion of acetone was followed in one experiment. There seems to have been no marked change as a result of the administration of the citrate.

It seems quite evident from these results that citric acid is capable of being quantitatively converted into glucose in the diabetic organism. It is, however, not necessary to assume that glucose is, normally, an intermediate product in the catabolism of citric acid. It appears to be more probable that at some stage in this process one or more substances are formed which may, under proper conditions, give rise to glucose. In order to account for the quantitative conversion of the carbon atoms of citric acid into glucose, it seems to be necessary to assume that the molecule of citric acid, either before or after reduction, breaks down into three molecules of a compound, or compounds, containing only two carbon atoms, which then recombine to form a straight six-carbon chain. Experiments to ascertain the nature of the intermediate products are now in progress.

SUMMARY.

The administration of sodium citrate to phlorhizinized dogs and to a patient with diabetes mellitus was followed by an increased excretion of glucose indicating the conversion of the six carbon atoms of citric acid into glucose.

EXPERIMENTAL.

Nitrogen was determined by the Kjeldahl method. Glucose was estimated by Benedict's method,⁴ checked by the polariscope. After fermentation the urines were slightly levorotatory and did not reduce Benedict's solution. In one experiment, the amount of glucose was also estimated by fermentation. The results agreed fairly well with those obtained by the other methods. Acetone and β -hydroxybutyric acid were estimated, in the case of J. L., by Shaffer's method.⁵ The figures given are not corrected.⁶ In the last experiment, acetone was determined by the Messinger-Huppert method.

⁴ Benedict: this *Journal*, ix, p. 57, 1911.

⁵ Shaffer: *ibid.*, v, p. 211, 1909.

⁶ Shaffer and Marriott: *ibid.*, xvi, p. 265, 1913.

TABLE I
Patient J. L.

DATE	FOOD		URINE					REMARKS
	Weight	N	Carbo- hydrate	N	Glucose		G:N	
	kilos	gms.	gms.	gms.	gross	net	gms.	
1913								
January								
27	44.27	17.1	21.0	15.49	78.8	57.8	3.73	40 gms. sodium citrate each day (= 20.2 gms. glucose).
28	42.45	12.3	24.1	13.26	72.5	48.4	3.65	
29	44.95	15.4	24.0	13.82	94.7	70.7	5.41	
							21.2	
30	46.10	15.5	15.1	11.87	76.9	61.8	5.21	50 gms. sodium citrate each day (= 25.2 gms. glucose). ("Extra glucose" Feb. 17-21 is 45.23 gms.)
31	47.67	13.8	13.1	12.08	57.7	44.6	3.73	
February							19.3	
1	48.58	21.2	21.3	14.48	67.9	46.6	3.22	
15	45.40	11.5	25.1	14.52	66.8	41.7	2.81	
16	44.83	16.4	24.0	11.55	54.0	30.0	2.60	
17	46.76	17.1	24.7	14.41	65.9	41.2	2.86	
18	47.90	15.1	24.2	13.10	63.0	38.8	2.96	
19	47.90	15.0	18.3	12.12	58.9	40.6	3.35	
20	47.67	13.5	22.1	10.17	66.8	34.7	4.19	
21	47.23	16.8	25.6	12.97	80.5	54.9	4.23	
22	45.65	16.2	25.5	11.91	59.8	34.3	2.88	
25	43.36	11.4	26.1	12.50	71.3	45.2	3.62	

In the experiments upon J. L., the food was carefully weighed and prepared in a special diet kitchen. So far as was possible, the patient's wishes regarding food were observed, so as to reduce the likelihood of his attempting to obtain some surreptitiously. He was watched carefully and it is believed that he did not obtain any other food during the experiments here reported. The figures given in the table for the nitrogen and carbohydrate in the food were calculated from those in Bulletin 28, U. S. Department of Agriculture. The figures in the column headed "net glucose" were obtained by subtracting the amount of carbohydrate in the food from the amount of glucose in the urine.

The dogs received 1 gram of phlorhizin in olive or cottonseed oil daily. They were catheterized at twelve-hour intervals and the bladder washed with sterile 2 per cent boric acid solution. The sodium citrate was given in 20 per cent solution, carefully sterilized. No infection was observed in any case.

TABLE II.

Phlorhizin dog 1.

PERIOD	NITROGEN	GLUCOSE	G:N	"EXTRA GLUCOSE"	REMARKS
	<i>grams</i>	<i>grams</i>		<i>grams</i>	
I	4.693	14.66	3.12		
II	4.883	16.03	3.28		
III	4.672	15.54	3.33		
IV	3.477	16.33	4.70	5.13	Weight 8.52 kilos. 16 gms. sodium citrate (= 8.07 gms. glucose) in two doses
V	4.970	16.70	3.36		
VI	4.702	14.20	3.02		
VII	3.700	18.47	4.99		
				11.38	Weight 8.18 kilos. 24 gms. sodium citrate (= 12.1 gms. glucose) in three doses.
VIII	3.220	15.26	4.73		
IX	3.849	12.47	3.24		
X	3.940	12.98	3.30		

TABLE III.
Phlorhizin dog 5.

PERIOD	NITROGEN	GLUCOSE	G:N	"EXTRA GLUCOSE"	REMARKS
	<i>grams</i>	<i>grams</i>		<i>grams</i>	
I	3.300	10.20	3.09	4.89	Weight 6.0 kilos. 12 gms. sodium citrate (=6.05 gms. glucose) in two doses.
II	3.420	9.68	2.83		
III	3.079	12.38	4.02		
IV	3.317	11.12	3.35		
V	3.275	9.35	2.86		
VI	3.090	9.91	2.88		

TABLE IV.
Phlorhizin dog 6.

PERIOD	NITRO- GEN	GLU- COSE	G:N	"EXTRA GLU- COSE"	ACE- TONE	REMARKS
	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>mgms.</i>	
I	9.070	35.97	3.96	18.50	26	Weight 15.3 kilos. 31.5 gms. sodium citrate (=15.9 gms. glucose) in two doses.
II	8.826	31.46	3.56		54	
III	7.382	40.27	5.46		79	
IV	8.404	37.43	4.45		581	
V	9.670	36.88	3.81	15.68	638	13 hours.
VI	7.944	29.10	3.66		773	11 hours.
VII	6.546	32.58	4.98		564	Weight 14.4 kilos. 32 gms. sodium citrate (=16.13 gms. glucose) in two doses.
VIII	5.554	21.94	3.95		728	
IX	7.773	21.72	2.76		868	
X	7.683	20.10	2.62		748	

ON CHONDROITIN SULPHURIC ACID.

THIRD PAPER.

By P. A. LEVENE AND F. B. LA FORGE.

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By the results of our previous work it was demonstrated that two carbohydrate derivatives occur in chondroitin sulphuric acid.¹ One was recognized as *d*-glucuronic acid and the other as a hexosamine. The hexosamine, in the form of its crystalline salt of hydrochloric acid, was obtained directly on concentration of the product of hydrolysis of chondroitin sulphuric acid. The specific rotation of this salt, measured in 1 per cent solution at room temperature and in pure yellow light, was found to be 99°. There was at the time only one hexosamine known, glucosamine, which is best identified as the hydrochloric acid salt. The initial specific rotation of this salt is given as + 100°. Hence, it was assumed that the hexosamine obtained from chondroitin sulphuric acid was identical with glucosamine.

Since then, the properties of the substance were scrutinized in greater detail, and the new information compels us to abandon the view of the identity of the new hexosamine with glucosamine. The substance is isomeric with glucosamine, and until the details of its structure will be disclosed it will be referred to as chondrosamine. Chondrosamine resembles glucosamine in its elementary composition and differs from it in the physical properties of its derivatives. A comparative study was made of the following derivatives: the hydrochloric acid salts, the phenylosazones, the dicarboxylic acids obtained on oxidation of the amino sugars by means of nitric acid, and of the monocarboxylic acids obtained on oxidation with bromine.

¹ This *Journal*, xv, p. 69 and p. 155, 1913.

The *hydrochlorides* differ in their crystal form, in their solubility in water and in dilute alcohol, in their melting points, and their specific rotations. The salt of glucosamine is quite soluble in water, almost insoluble in 80 per cent alcohol; the salt of the new substance is quite soluble in 80 per cent alcohol. The salt of glucosamine showed no tendency to melt at a temperature considerably above $200^{\circ}\text{C}.$, whereas the melting point of the hydrochloride of chondrosamine is sharp at $182^{\circ}\text{C}.$ The optical rotation of the two substances is particularly worthy of note. That of the salt of chondrosamine has an initial specific rotation of 129° , is mutarotating and reaches the equilibrium at 93° ; glucosamine hydrochloride also is mutarotating, with the initial rotation of 100° and the equilibrium point at 73° .

If the rotation of chondrosamine is measured at room temperature and in 1 per cent solution it may show the effect of mutarotation already at the time of the first reading. This occurred in our first experiments with the substance, and the occurrence misled us into the belief that chondrosamine possessed the specific rotation of glucosamine. In order to avoid this possible error the first reading should be taken immediately after solution of the substance is effected and precautions should be exercised to prevent the temperature of the solution from rising above $0^{\circ}\text{C}.$

The *osazones* differ in their melting points, and in their specific rotation. The phenylosazone of glucosamine is regarded as identical with glucosazone. A sample prepared by us had a melting point of $208^{\circ}\text{C}.$, possessed the solubility of glucosazone and hence could be recrystallized out of 98 per cent alcohol. The initial rotation under the conditions indicated by Neuberg was -0.62° and the equilibrium -0.35° . The phenylosazone of chondrosamine is very soluble even in dilute alcohol and has to be recrystallized out of water. The melting point of the substance is depressed apparently by very slight impurities. The highest melting point was obtained only when measured on a very fresh sample of the substance, that had not been exposed to the light. The highest melting point obtained was 180° with decomposition at rapid heating.

It must be noted also that chondrosamine forms apparently the same osazone if it had been deaminized prior to the treatment

with phenylhydrazine. According to Fischer and Tiemann,² glucosamine if deaminized yields only an insignificant quantity of osazone, and Tiemann assumed that this was due to the fact that deaminization was incomplete. The melting point of the osazone obtained from deamino-chondrosamine showed the identical melting point with the substance obtained directly from the amino sugar. However, this was attained only by repeated recrystallization. After one recrystallization the substance melts at about 160°C. and shows a decomposition point at 180°C.

The initial rotation of two samples of the osazone, one formed directly from the sugar and the other from the deamino sugar, showed a discrepancy, but the equilibrium rotation was practically identical and both different in magnitude and in direction from that of glucosazone: namely, the initial rotation was $+0.60^\circ$ for the first and $+0.45^\circ$ for the second, the equilibrium rotations being $+0.20$ and $+0.18^\circ$ respectively.

The dicarboxylic acids were analyzed in form of their calcium salts. The most striking difference was found in their specific rotation. Isosaccharic acid prepared by us showed an initial rotation of $+0.20^\circ$ and final rotation of $+0.06^\circ$. The acid obtained from the new sugar showed an initial rotation of 0.45° and final of 0.37° . The acid will be referred to as "chondrosic acid."

Monocarboxylic acid of the new sugar will be referred to as chondronic acid. It was obtained and analyzed in form of its brucine salt. The substance melted at 213° and had a specific rotation of 16.85° . No corresponding salt of chitonic acid has been described.

Discussion of results. The results here enumerated permit only of the conclusion that chondrosamine is isomeric with glucosamine. The fact that in the process of the osazone formation the amino group is eliminated from the molecule, renders it probable that the group is attached to the α -carbon (to the carbonyl group). The details of the structure of chondrosamine will be established by further investigation. Work in this direction is now in progress.

In conclusion we wish to refer again to an observation made in Hofmeister's laboratory. Kondo³ prepared on treatment of the

² Ber. d. d. chem. Gesellsch., xxvii, p. 140, 1894.

³ Biochem. Zeitschr., xxvi, p. 116, 1910.

products of hydrolysis of chondroitin sulphuric acid an osazone having a melting point at 143°C . Discussing this observation Kondo referred to the similarity of the osazone with that of xylose. We inferred that the discussion indicated the author's inclination to regard xylose as a probable constituent of chondroitin sulphuric acid. Since then Professor Hofmeister informed us in private correspondence that both he and his co-workers were convinced that the similarity of their osazone with xylosazone was only superficial, and that we attached undue emphasis to their discussion. At present it seems to us probable that their osazone was that of chondrosamine, but in a state of imperfect purity.

EXPERIMENTAL.

Preparation of hexosamine hydrochloride.

Seventy-five grams of chondroitin sulphuric acid barium salt were hydrolyzed for about seven and a half hours with 400 cc. of 20 per cent hydrochloric acid, with the addition of 15 grams of stannous chloride. Barium sulphate began to separate at once and soon the solution began to take on a yellow color which passed rapidly through brown to black with the separation of dark particles due to decomposition of glucuronic acid. Upon completion of the reaction the solution was diluted with twice its volume of warm water and without filtering the tin removed with hydrogen sulphide. The sulphides of tin were separated by filtration with suction, leaving a clear, almost colorless filtrate which, without further treatment, was concentrated in vacuum to about 35 cc. This syrup-like residue was at once taken up in 75 to 80 cc.⁴ of absolute alcohol, poured into a beaker and the hydrochloride of the amino hexose caused to crystallize by adding about 100 cc. of absolute ether slowly in portions of about 10 cc. with constant scratching of the sides of the vessel. The deposit of long white prismatic needles thus obtained was filtered with suction and washed with absolute alcohol and ether. The first yield usually amounts to about 16 grams while upon addition of 50 cc. more of ether to the first filtrate about 4 grams more of equally pure

⁴ The presence of too much alcohol or water causes the product to separate oily at first.

product are obtained. The total yield corresponds to about 90 per cent of the theory. Upon recrystallization under the above conditions or on long keeping in a desiccator, the product tends to lose a small amount of hydrochloric acid, since an analysis of a product twice so treated gave the following figures:⁵

0.1794 gram of substance required 14.6 cc. AgNO_3 solution (1 cc. = 0.00186 gram Cl):

0.1701 gram of substance gave 0.0977 gram H_2O and 0.2113 gram CO_2 .

	Calculated for $\text{C}_6\text{H}_{15}\text{O}_5\text{N.HCl}$:	Found:
Cl.....	16.45	15.15
C.....	33.40	33.93
H.....	6.54	6.55

The optical determination was carried out at 0° . All apparatus was cooled to this temperature. 0.3000 gram of substance in 3 cc. H_2O , total weight of solution 3.2871 grams, specific gravity 1.0352.

After five minutes + 6.10° : $[\alpha]_D^{20} = 129.50^\circ$.

After twenty-four hours + 4.44° : $[\alpha]_D^{20} = 93.82^\circ$.

The activity of pure glucosamine was determined for comparison under the same condition. 0.3000 gram of substance in 3 cc. H_2O , weight of solution 3.2876 grams, specific gravity 1.0327.

After five minutes + 4.79° : $[\alpha]_D^{20} = 101.60^\circ$.

After twenty-four hours + 3.50° : $[\alpha]_D^{20} = 73.65^\circ$.

Phenylosazone from deamino chondrosamine.

Three grams of the hexosamine hydrochloride were deaminized with 3 grams of silver nitrite with the addition of a few drops of hydrochloric acid. After about three hours, at room temperature, the silver chloride was filtered off and an excess of silver in the filtrate removed by treatment with hydrogen sulphide. The solution was then boiled to remove the excess of the latter and heated for one and a half hours on the water bath with 6 grams of phenylhydrazine in 20 cc. of glacial acetic acid and 3 grams of sodium acetate. It was then diluted to 300 cc. with hot water and filtered. Upon cooling the osazone deposits in the filtrate in long yellow needles which were after a time filtered off and recrystallized from

⁵ Normal figures are obtained if the substance is crystallized from alcohol and ether containing a small amount of free hydrochloric acid (this *Journal*, xv, p. 155, 1913).

300 cc. of water. The yield of the first product was over 1 gram. The substance melts at about 160° and decomposes at about 180° . Again recrystallized the melting point rose to 175° with decomposition at about 185° . Upon keeping, especially if not quite pure, the product deteriorates rapidly with superficial decoloration.

0.1212 gram of substance gave 0.0650 gram of H_2O and 0.2697 gram of CO_2 .

	Calculated for $C_{15}H_{21}N_4O_7$	Found:
C.....	60.33	60.76
H.....	6.14	6.01

The rotation was determined in Neuberg's pyridine-alcohol mixture in 0.5 dm. tube with D-light. 0.1000 gram of substance in 5 cc. rotated after 18 hours $+ 0.20^{\circ}$.

Phenylosazone from chondrosamine.

Three grams of chondrosamine hydrochloride were dissolved in 100 cc. of water to which the calculated amount of sodium acetate was added. The solution was warmed in the boiling water bath and to the warm solution was added the required amount of phenylhydrazine dissolved in 5 to 8 cc. of glacial acetic acid. The flask containing the solution and provided with a return condenser was heated in a boiling water bath for about three hours, and the reaction product was allowed to cool. On cooling the osazone separated in form of a flocculent mass. It was recrystallized out of hot water. The dry precipitate was washed very carefully with ether. Two recrystallizations suffice to furnish a pure, bright yellow osazone.

The melting point of the osazone dried in a vacuum desiccator over sulphuric acid was at $180-185^{\circ}C$.

0.1000 gram of the substance was dissolved in 5 cc. of Neuberg's pyridine-alcohol mixture and showed at pure yellow light after 18 hours' standing, α in 0.5 dm. tube at 20° , $+ 0.18^{\circ}$.

0.1280 gram substance gave 18 cc. N at 25° , 765 mm.

	Calculated:	Found:
N.....	15.64	15.52

Nitric acid oxidation of hexosamine.

Nine grams of hexosamine hydrochloride were deaminized as in the preceding experiment with silver nitrite, the resulting solution concentrated to about 20 cc., mixed with an equal volume of con-

concentrated nitric acid and allowed to stand over night at 42°. It was then rapidly evaporated in a shallow dish on a water bath and the syrup, after having been again evaporated with water, was diluted to 250 cc., and boiled with calcium carbonate until neutral (one-half hour). The filtrate, upon standing for two days, deposited white prisms of the calcium salt of a dibasic hexonic acid. The yield did not exceed 25 per cent of the theory. For analysis it was recrystallized by dissolving in 50 parts of boiling water containing slightly over the theoretical amount of oxalic acid and again transformed into the calcium salt by boiling with calcium carbonate. The compound contained 2 molecules of crystal water which can be removed in vacuum at 108°. By heating for sixteen hours at 138° no further appreciable loss of weight was observed.

The dried substance analyzes best for the calcium salt of a normal dibasic hexonic acid.

0.1504 gram of air-dried substance gave 0.0200 gram H₂O (108°).

0.1508 gram of air-dried substance gave 0.0208 gram H₂O (108°).

0.1509 gram of air-dried substance gave 0.0198 gram H₂O (108°).

0.1670 gram of air-dried substance gave 0.0232 gram H₂O (140°).

	Calculated for C ₆ H ₁₀ O ₆ Ca+2H ₂ O:	I	Found: II	III	IV
H ₂ O.....	12.68	13.30	13.06	13.13	13.28
0.1304 gram dried substance gave 0.0344 gram H ₂ O, 0.1376 gram CO ₂ and 0.0302 gram CaO.					
0.1312 gram of dried substance gave 0.0330 gram H ₂ O, 0.1380 gram CO ₂ and 0.0302 gram CaO.					
0.1030 gram of dried substance gave 0.0296 gram H ₂ O, 0.1083 gram CO ₂ and 0.0243 gram CaO.					

	Calculated for C ₆ H ₁₀ O ₆ Ca:	I	Found: II	III	IV
C.....	29.03	28.78	28.68	28.67	
H.....	3.22	2.95	3.24	3.22	
CaO.....	22.58	23.36	21.80	23.15	22.50

0.1020 gram of substance in 2 cc. 10 per cent HCl rotated in 1 dm. tube after 10 minutes, -0.45°; after 18 hours, -0.37°.

Isosaccharic acid calcium from glucosamine. 0.0786 gram of substance in 2 cc. 10 per cent HCl rotated in 1 dm. tube after 10 minutes, +0.20°; after 18 hours, +0.06°.

Brucine salt of hexonic acid.

Eighteen grams of hexosamine hydrochloride were deaminized with silver nitrite and the excess of silver removed in this case with a slight excess of hydrochloric acid, and the solution (volume, 150 cc.) allowed to stand for three days with bromine added in portions so that it was always present in excess. The reaction product was after this time freed from bromine by distillation in vacuum and the hydrobromic acid removed with silver carbonate. An excess of silver in the filtrate was removed with hydrogen sulphide and the solution warmed on the water bath with 45 grams of brucine. It was then cooled and filtered from the excess of the latter and concentrated to a syrup which crystallized to a semisolid cake. This was extracted with cold alcohol, filtered, washed and recrystallized from about 100 to 150 parts of hot absolute alcohol. The first product crystallized in thin plates which are fairly easily soluble in hot alcohol, changing upon recrystallization to short heavy prisms which are then only soluble with difficulty. The product crystallized from 98 per cent alcohol seemed to contain 1 molecule of crystal water which could be removed by heating in vacuum at 100°. It analyzes best for the brucine salt of an anhydrohexonic acid.

0.1190 gram of substance gave 0.0034 gram H_2O .

0.1156 gram of substance gave 0.0640 gram H_2O and 0.2564 gram CO_2 .

	Calculated for $\text{C}_{27}\text{H}_{35}\text{N}_2\text{O}_{10} + \text{H}_2\text{O} (590.4)$:	Found:
C.....	60.80	60.49
H.....	6.10	6.20
H_2O	3.11	2.86

0.3000 gram of substance in 3 cc. H_2O , weight of solution, 3.2825 grams, rotated in 0.5 dm. tube at 20° and D-light + 0.77°.

$$[\alpha]_D^{20} = 16.85.$$

THE NON-INTERFERENCE OF 'PTOMAINES' WITH CERTAIN TESTS FOR MORPHINE.

SECOND COMMUNICATION.

By JACOB ROSENBLOOM.

(From the Biochemical Laboratory of the Western Pennsylvania Hospital,
Pittsburgh, Pa.)

(Received for publication, May 5, 1914.)

A short time ago¹ I reported with Dr. S. R. Mills the results of certain experiments showing that bacterial products formed during aerobic and anaerobic putrefaction of certain human organs did not in any way give reactions simulating those due to the presence of morphine and in no way interfered with the detection of morphine when morphine was added to these putrefactive products.

I now wish to report the result of some work confirming the observations mentioned. I recently had occasion to exhumate a body thirteen months after burial. There were certain reasons to think that this person may have been poisoned by morphine. The brain, lungs, stomach, liver, heart-muscle and thigh-muscle were taken for examination. To about one-third of this material 100 mgms. of morphine sulphate was added. Both portions were then carried through the Dragendorff extraction, as described by Witthaus.²

The extract of the portion to which no morphine was added on testing by means of the following reactions, ferric chloride, Fröhde's, Pellagri's, Husemann's, nitric acid and iodic acid failed to show any reaction that might be mistaken for the reactions that morphine gives with the above mentioned tests. The extract of the portion to which the morphine was added gave these reactions

¹ This *Journal*, xvi, p. 327, 1913.

² Witthaus and Becker: *Medical Jurisprudence, Forensic Medicine and Toxicology*, 1911, iv, p. 999.

very clearly. From these results it may be noted that in certain organs removed from a body thirteen months after burial with marked putrefactive changes in the organs there was no difficulty encountered through the interference of the so-called "ptomaines" as regards their giving reactions simulating those due to morphine or by their presence interfering with the detection of morphine.

Since it has also been claimed that bacterial products may give reactions similar to those due to conine, nicotine, atropine, strychnine, digitalin, veratrine, colchicine, and delphinine I thought it would be of interest to examine the various extracts obtained in the Dragendorff method for these substances. No reactions that might be mistaken for those due to any of the above mentioned substances were obtained.

THE RATE OF DISAPPEARANCE OF AMMONIA FROM THE BLOOD IN NORMAL AND IN THYROIDECTOMIZED ANIMALS.

By CLARA JACOBSON.

(From the Hull Physiological Laboratory of the University of Chicago.)

(Received for publication, May 6, 1914.)

In an earlier study of the condition of the liver in parathyroid tetany,¹ we made use of the method of perfusing the excised liver and taking the rate of ammonia destruction as an index of liver function. It was clearly recognized that this method involves serious and unavoidable errors. In order to avoid these a repetition of the tests by the present method was undertaken at the request of Dr. Carlson. The method consists of determinations of the rate of disappearance of the ammonia from or in the circulating blood after intravenous injections of ammonium carbonate. The liver is left in its normal relations and the factor of elimination of ammonia through the kidney excluded by ligature of the renal vessels.

METHODS.

The blood ammonia was determined by practically the same method used in the previous work.

15 or 20 cc. of defibrinated blood were placed in a tall cylinder, the volume was made up to 25 cc. with distilled water, and 4 grams of NaCl and 2.5 grams of amorphous sodium carbonate were added (10-15 cc. of toluol were added to prevent excessive foaming). Air washed in sulphuric acid was forced through the blood for four hours, the ammonia liberated being caught in another tall cylinder containing 100 cc. of $\frac{N}{100}$ H₂SO₄. The amount of ammonia present was determined by Nessler's reagent. 15-25 cc. of the acid containing ammonia were diluted to 50 cc. and 1 cc. of reagent was

¹ A. J. Carlson and Clara Jacobson: *Amer. Journ. of Physiol.*, xxviii, p. 133, 1911.

added, and the color compared with a series of tubes containing known quantities of ammonia (0.005 to 0.05 mgm.). The amount in the whole 100 cc. of acid was calculated and then the quantity per 100 cc. of blood.

The first series of experiments was made on normal cats under light ether anesthesia. The sample of normal blood was taken from the carotid artery. The renal arteries and veins on both sides were then ligatured, and ammonium carbonate of about 1.5 per cent was injected fairly rapidly into the exposed femoral vein—in quantities varying from 0.06 to 0.09 gram per kilo of body weight. The second sample of blood was drawn five minutes after the injection. In some experiments a third sample was drawn fifteen minutes after the injections. The results are given in Table I.

TABLE I.

Normal cats.

Ammonia content of the blood before and five minutes after intravenous injections of ammonium carbonate solution. Renal vessels ligated.

NO. OF ANIMAL	(NH ₄) ₂ CO ₃ INJECTED	NH ₃ PER 100 CC. BLOOD		DIFFERENCE
		Before injection	5 min. after injection	
	<i>gm. per kilo</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
1	0.03	0.48	0.66	0.2
2	0.06	0.61	0.85	0.25
3	0.066	0.66	1.40	0.75
4	0.057	0.64	0.72	0.1
5	0.06	0.72	1.1	0.4
6	0.066	0.61	0.95	0.35
7	0.042	0.53	0.62	0.1
Averages.....	0.054	0.60	0.9	0.3

In a second series of experiments the above methods were repeated with thyroparathyroidectomized animals showing tetany. These results are recorded in Table II.

In a third series normal cats were anesthetized and the carotid was prepared for withdrawing blood. Through a median incision in the abdomen, the renal vessels were ligated and all the arteries which could be found going off from the aorta to the gastro-intestinal tract, and lastly, to make sure the liver was out of circulation, the hepatic arteries and portal vein were included in a liga-

TABLE II.

*Cats in parathyroid tetany.**Ammonia content of the blood before and five minutes after an intravenous injection of ammonium carbonate. Renal vessels ligated.*

NO. OF ANIMAL	(NH ₄) ₂ CO ₃ INJECTED	NH ₃ PER 100 CC. BLOOD		DIFFERENCE
		Before injection	5 min. after injection	
	gm. per kilo	mgm.	mgm.	mgm.
1	0.08	0.53	0.76	0.23
2	0.06	0.9	1.3	0.4
3	0.05	0.75	1.3	0.55
4	0.05	1.1	1.7	0.6
5	0.09	1.0	1.4	0.4
6	0.09	0.8	1.4	0.6
Averages.....	0.07	0.85	1.31	0.46

ture. In this way we attempted to determine how much of the disappearance of the ammonia from the blood might be due to liver activity. The gastro-intestinal tract was also eliminated from the circulation because if the vessels to the liver alone were ligated the stasis in the portal circulation would of itself produce abnormal conditions. The results are given in Table III.

TABLE III.

*Normal cats.**Ammonia content of the blood before and five minutes after injections of ammonium carbonate solution. The blood vessels to kidney, stomach, intestines, and liver were ligated before the injection.*

NO. OF ANIMAL	(NH ₄) ₂ CO ₃ INJECTED	NH ₃ PER 100 CC. BLOOD		DIFFERENCE
		Before injection	5 min. after injection	
	gm. per kilo	mgm.	mgm.	mgm.
1	0.08	0.08	7.3	6.42
2	0.08	1.2	4.4	3.2
3	0.06	1.0	3.0	2.0
4	0.06	1.2	5.62	4.42
5	0.06	1.2	9.1	7.9

RESULTS.

Considering the relatively large quantity of ammonia injected into the blood the analyses indicate a very rapid disappearance of the excess from the blood in both normal and thyroidectomized animals. The average ammonia content of blood of seven normal cats was 0.6 mgm. per 100 cc. as against 0.9 mgm. five minutes after the injection of 50 mgm. per kilo body weight. There was noted, also, considerable individual variations in the amounts which remained after five minutes. In the thyroidectomized animals the ammonia content averaged 0.85 mgm. per 100 cc. of blood, with 1.31 mgm. five minutes after the injections. There seems to be a slightly greater ammonia content in the blood of the thyroidectomized animals, and a slight decrease in the rate of disappearance of the ammonia from the blood. The differences, however, are so small as to be of doubtful significance.

The third series of experiments (Table III) gives an indication of the amount of ammonia that disappears from the blood by diffusion into tissue spaces, lymph, and cells outside the gastrointestinal tract. These animals showed an increase in susceptibility to the ammonia injections—not a few having marked symptoms. The analyses show a much greater excess of ammonia than in the previous series I and II.

In view of the fact that these animals received 100 or more mgm. of ammonia per 100 cc. of blood intravenously, the presence of as little as 3 to 9 mgm. per 100 cc. of blood five minutes after the injection means that nearly all the ammonia passes out of the blood quite apart from any action of the kidneys, the liver, or the digestive tract. The larger excess remaining in the last series of experiments may be due mainly to the exclusion of so much tissue (the liver, stomach, and intestines) from the circulation rather than a depression of the ammonia-destroying function of the liver.

SUMMARY.

1. Even with the kidneys excluded from the circulation, ammonia injected into the blood is so rapidly removed from the blood that only a very slight excess is present five minutes after the injection.

2. There is only a very slight difference between the normal and parathyroidectomized animal in the rate of disappearance of the ammonia from the blood.

3. The method employed is not adequate for the determination of liver efficiency (ammonia-destroying power), because the rate of disappearance of excess of ammonia from the blood independent of the liver function is so great as to render the actual ammonia destruction by the liver almost a negligible factor.

THE BASAL, GASEOUS METABOLISM OF NORMAL MEN AND WOMEN.¹

BY FRANCIS G. BENEDICT, LOUIS E. EMMES, PAUL ROTH,
AND H. MONMOUTH SMITH.

*(From the Nutrition Laboratory of the Carnegie Institution of
Washington, Boston, Mass.)*

(Received for publication, May 9, 1914.)

The impetus given to the study of gaseous and gross metabolism during the past decade has resulted in a large number of observations, both in the domain of physiology and pathology. Investigators in pathology are, however, continually confronted by the paucity of normal data with which to compare their observations. Benedict and Joslin,² stimulated by the arrival in Boston of Dr. Falta of Vienna, began immediately studying the metabolism of diabetics with the new respiration calorimeters in the Nutrition Laboratory. Before their investigation was completed the need for normal data for control was imperative. Though they recognized the importance of normals and published in their first publication on diabetics a number of normals for comparison, Lusk³ pointed out the deficiency in the number of normals that they

¹ The larger number of observations reported here were made in the Nutrition Laboratory, chiefly by Emmes, but in part by T. M. Carpenter and H. L. Higgins, in connection with other researches. We wish here to acknowledge our obligation to Messrs. Carpenter and Higgins.

Certain of the experiments were made on vegetarians by Roth at Battle Creek, Michigan, in the Battle Creek Sanitarium. Our thanks are due to Dr. J. H. Kellogg for providing the respiration apparatus and the subjects for the experiments on vegetarians.

The experiments on athletes were carried out by Smith, in the Chemical Laboratory of Syracuse University, Syracuse, New York.

In the tabular classification of the experiments the name of the experimenter will accompany each observation.

² Benedict and Joslin: *Metabolism in Diabetes Mellitus*, Carnegie Institution of Washington, Publication No. 136, 1910.

³ Lusk: *Science*, xxxiii, p. 433, 1911.

reported, and hence in their second diabetic publication⁴ they enlarged the list of normal individuals for comparison purposes.

More recently a visit by two of us to the Respiration Calorimeter Laboratory in Bellevue Hospital, New York City, brought out the fact that Drs. Coleman and DuBois were making an extensive and fundamental study of metabolism during typhoid fever, using an extremely accurate bed calorimeter, and we were there again impressed with the fact that there was a great deficiency of knowledge of the metabolism of normal individuals available for the purposes of comparison. With infants, Benedict and Talbot⁵ have experienced a similar difficulty. However, within the last year or two the increased use of the respiration calorimeter of the type developed at Wesleyan University and in this Laboratory, and of the universal respiration apparatus has made possible the accumulation of a considerable number of normal as well as pathological values.

Since by far the larger amount of work on normal individuals has been carried out in a coördinate series of experiments in connection with this laboratory and others which we represent, it has been felt desirable to prepare immediately a summary of the results on normal individuals obtained with these forms of apparatus up to the present date.

In dealing with normal men difficulties are always experienced when the conception of the word "normal." As popularly understood "normal" would mean "average," but in dealing with metabolism observations we may draw a somewhat sharper line and say "average values obtained from people in presumably good health." It has been clearly shown that gaseous metabolism is profoundly affected by a number of factors—muscular activity and fever being most pronounced in their effects. By selecting persons of normal temperature, or at least of non-febrile temperature, during periods of muscular repose these two factors may be eliminated. But with every individual there are periodicities in the rate of metabolism during the day, incidental to the ingestion

⁴ Benedict and Joslin: *A Study of Metabolism in Severe Diabetes*, Carnegie Institution of Washington, Publication No. 176, 1912.

⁵ Benedict and Talbot: *The Gaseous Metabolism of Infants, with Special Reference to its Relation to Pulse-rate and Muscular Activity*, Carnegie Institution of Washington, Publication No. 201, 1914.

of food. It has been shown that when a mixed diet is taken, the ingestion of food almost invariably produces an increase in metabolism. This has been variously ascribed to the increased work of digestion, to the specific, dynamic action of the foodstuffs, and to the specific, catabolic stimuli, absorbed from the food in its passage through the alimentary tract.

It is obvious, therefore, that with varying diets, particularly when the amount of protein is varied, the effect of this stimulus may vary enormously, and while it is, theoretically at least, not impossible to adjust a standard diet so that each individual will be stimulated approximately alike, it has been found that it is best to attempt in all fundamental measurements of metabolism to eliminate this uncertain factor of the specific, catabolic stimuli by insisting as a prerequisite of all experiments that the subject should not be examined unless twelve hours, or more, have elapsed since the last meal, or what we prefer to state as in the *post-absorptive* condition.

Another factor, or complexity of factors, may not be so sharply differentiated, but the index, which is the pulse rate, shows that there is a clearly established relationship between the pulse rate and the total metabolism. This, while being more marked with some individuals than with others, is nevertheless almost invariably to be observed. If subject A, in a resting, post-absorptive condition, has on a given day a pulse rate of 70 per minute, and on a subsequent day under exactly the same conditions has a pulse rate of 60 per minute, it may be asserted with every degree of confidence that the metabolism on the second day will be perceptibly, indeed measurably, lower than on the first.

Under these conditions, therefore, it can be seen that to attempt to secure the minimum normal metabolism of any given individual, even excluding the influence of muscular activity or the ingestion of food, should theoretically require the measure of metabolism to be made during the period when the subject was having the minimum pulse rate.

The body temperature, even though slightly above normal, may affect considerably the total metabolism, and while in experiments reported here, where the temperatures of the mouth are recorded, occasionally temperatures as high as 99.1° F. (37.28°C.) or 99.2° F. (37.34°C.) have been accepted, it can be safely asserted that

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TABLE 1

Metabolism of

SUBJECT	AGE	OBSERVATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL PER MIN.	
		Days	Periods			Carbon dioxide	Oxygen
	<i>years</i>			<i>kilos</i>	<i>cm.</i>	<i>cc.</i>	<i>cc.</i>
H. W.....	19	1	2	108.9	198	326	361
W. S.....	22	3	6	88.5	165	241	289
O. F. M.....	24	2	6	85.8	171	209	265
F. G. B.....	41	8	37	83.1	183	217	258
Prof. C.....	36	3	12	83.0	169	199	237
J. H. R.....	23	3	6	82.2	187	243	282
D. H. W.....	22	3	4	82.1	186	245	291
H. F.....	63	1	4	82.1	166	180	236
M. H. K.....	19	2	3	79.0	188	243	276
E. G.....	20	6	11	78.9	184	262	302
W. A. M.....	23	2	6	78.0	183	213	262
Dr. M.....	28	2	12	75.9	180	214	273
M. Ba.....	20	1	3	75.0	180	221	263
F. E. M.....	38	2	6	75.0	164	209	242
J. F. M.....	20	1	2	74.5	181	227	269
F. A. R.....	32	3	13	74.4	163	205	244
W. J. T.....	22	4	13	74.2	183	206	256
F. G. R.....	20	4	8	74.0	179	242	267
C. D. R.....	22	3	6	74.0	173	238	270
H. R. W.....	24	3	6	73.9	175	222	264
J. P. C.....	23	4	14	73.7	169	186	218
A. O. G.....	25	1	4	73.2	179	194	271
H. W. E.....	23	2	8	73.0	168	190	222
P. D. F.....	23	3	6	71.2	176	219	259
C. B. S.....	26	26	75	71.1	179	202	244
J. H. H.....	25	5	13	69.1	171	197	234
Dr. N. K. W...	35	1	3	68.4	166	196	219
B. A. W.....	26	2	7	67.9	174	229	280
K. H. A.....	26	25	110	66.4	182	194	238
J. R.....	27	12	58	66.0	182	201	241
M. A. M.....	29	53	157	66.0	177	206	242
F. P. R.....	22	20	58	65.1	173	182	222
J. J. C.....	27	53	252	65.0	175	190	227
E. H. T.....	25	2	7	64.7	170	173	217
D. M.....	22	5	15	64.0	171	187	240
M. J. S.....	24	13	42	63.7	170	195	237
M. Y. B.....	20	6	12	63.5	172	207	238
R. D. S.....	21	3	6	63.5	170	205	228

normal men.

PER KILO PER MIN.		PULSE RATE	HEAT PER 24 HRS. (CALCULATED)			OBSERVER
Carbon dioxide	Oxygen		Total	Per kilo of body weight	Per square meter of body surface	
cc.	cc.		cals.	cals.	cals.	
2.99	3.31	71	2559	23.5	911	Smith.
2.72	3.27	54	2017	22.8	823	Smith.
2.44	3.09	57	1827	21.3	761	Emmes.
2.61	3.10	65	1802	21.7	770	Carpenter.
2.40	2.85	61	1655	19.9	707	Emmes.
2.95	3.43	65	1978	24.1	849	Smith.
2.99	3.55	58	2034	24.8	873	Smith.
2.19	2.90	68	1615	19.7	693	Carpenter.
3.07	3.49	67	1944	24.6	856	Smith.
3.32	3.83	59	2126	27.0	940	Smith.
2.73	3.36	65	1816	23.3	807	Emmes.
2.82	3.60	58	1877	24.7	849	Higgins.
2.95	3.51	63	1837	24.5	839	Carpenter.
2.79	3.22	55	1698	22.7	775	Roth.
3.05	3.61	67	1878	25.2	861	Higgins.
2.76	3.28	57	1704	22.9	782	Carpenter.
2.78	3.45	60	1770	23.9	816	Carpenter.
3.27	3.61	61	1914	25.9	882	Smith.
3.22	3.65	63	1908	25.8	879	Smith.
3.00	3.57	63	1842	24.9	848	Smith.
2.52	2.96	53	1526	20.7	706	Emmes.
2.65	3.70	57	1835	25.1	853	Carpenter
2.60	3.04	49	1559	21.4	725	Carpenter.
3.07	3.64	54	1810	25.4	858	Smith.
2.84	3.43	63	1700	23.9	806	Higgins.
2.85	3.39	63	1634	23.6	789	Carpenter.
2.87	3.20	50	1549	22.6	752	Emmes.
3.37	4.12	59	1945	28.6	949	Higgins.
2.92	3.58	49	1654	24.9	819	Carpenter.
3.05	3.65	64	1679	25.4	835	Emmes.
3.12	3.67	62	1695	25.7	843	Cathcart.
2.80	3.41	59	1543	23.7	775	Higgins.
2.92	3.49	60	1585	24.4	796	Carpenter.
2.67	3.35	70	1499	23.2	757	Roth.
2.92	3.75	61	1651	25.8	838	Higgins.
3.06	3.72	61	1647	25.9	840	Carpenter.
3.26	3.76	62	1677	26.4	856	Smith.
3.23	3.60	57	1619	25.5	826	Smith.

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TABLE 1—Continued

Metabolism of

SUBJECT	AGE	OBSERVATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL PER MIN.	
		Days	Periods			Carbon dioxide	Oxygen
	<i>years</i>			<i>kilos</i>	<i>cm.</i>	<i>cc.</i>	<i>cc.</i>
E. P. C.....	35	3	12	63.2	185	171	216
R. G.....	23	4	16	62.7	173	194	227
U. R. B.....	27	1	3	62.6	173	179	220
W. F. M.....	21	7	12	62.4	180	221	259
H. H. A.....	22	28	81	62.3	164	179	213
H. C. B.....	27	2	4	62.0	173	192	239
S. A. R.....	23	9	44	60.8	165	175	209
A. L.....	40	4	12	60.6	171	191	225
W. G. J.....	21	9	26	60.5	175	210	250
H. L. H.....	26	35	120	60.5	172	200	244
J. E. F.....	21	7	27	60.4	172	202	229
J. K. M.....	24	27	103	60.4	173	182	223
H. B. R.....	25	2	10	60.2	168	173	214
J. B. T.....	20	11	44	60.1	171	209	251
W. F. B.....	32	5	16	60.1	168	199	233
L. H. W.....	27	1	3	60.0	179	184	219
H. B. L.....	20	5	18	60.0	173	190	229
L. E. E.....	31	31	144	59.8	175	204	245
F. M. M.....	16	4	18	59.7	173	203	251
W. B. L.....	29	1	3	59.3	164	165	211
T. H. Y.....	22	2	6	59.2	169	190	231
Dr. S.....	43	5	13	58.5	181	153	193
B. K.....	39	1	3	58.2	178	166	200
D. J. M.....	20	5	31	58.0	175	189	233
H. F. T.....	32	41	211	57.8	179	165	192
E. T. W.....	22	4	12	57.8	169	171	213
P. F. J.....	20	18	82	57.2	167	193	232
L. D. A.....	19	2	6	57.1	171	188	220
A. G. E.....	26	14	68	57.0	169	195	216
R. I. C.....	26	2	9	56.8	184	194	244
C. J. D.....	27	3	6	56.7	160	—	218
J. W. P.....	30	4	15	56.5	172	203	243
W. W. C.....	17	2	4	56.3	172	199	232
W. A. S.....	21	5	10	56.3	169	190	223
J. C. C.....	22	2	7	56.1	173	179	219
Dr. P. R.....	41	9	33	52.2	164	158	193
O. N. A.....	25	1	3	55.4	171	177	224
C. H. H.....	19	9	25	55.1	169	173	203
I. A. F.....	24	3	18	54.9	156	190	232

normal men.

PER KILO PER MIN.		PULSE RATE	HEAT PER 24 HRS. (CALCULATED)			OBSERVER
Carbon dioxide	Oxygen		Total	Per kilo of body weight	Per square meter of body surface	
cc.	cc.		cals.	cals.	cals.	
2.71	3.42	49	1489	23.6	764	Carpenter.
3.09	3.62	63	1590	25.4	820	Carpenter.
2.86	3.51	52	1525	24.4	786	Higgins.
3.54	4.16	78	1816	29.1	936	Smith.
2.87	3.42	66	1487	23.9	770	Higgins.
3.10	3.85	59	1653	26.7	856	Higgins.
2.88	3.44	53	1460	24.0	768	Carpenter.
3.15	3.71	73	1576	26.0	829	Carpenter.
3.47	4.13	70	1746	28.9	919	Higgins.
3.31	4.03	68	1696	28.0	893	Emmes.
3.34	3.79	57	1616	26.8	851	Cathcart.
3.01	3.69	57	1549	25.6	815	Carpenter.
2.87	3.55	67	1487	24.7	787	Higgins.
3.48	4.18	65	1748	29.1	925	Carpenter.
3.31	3.88	54	1632	27.2	863	Carpenter.
3.07	3.65	57	1530	25.5	810	Roth.
3.17	3.82	65	1596	26.6	844	Carpenter.
3.41	4.10	58	1707	28.5	908	Riche.
3.40	4.20	50	1739	29.1	925	Carpenter.
2.78	3.56	70	1451	24.5	776	Roth.
3.21	3.91	66	1605	27.2	861	Roth.
2.62	3.30	56	1331	22.8	716	Higgins.
2.85	3.44	—	1393	23.9	753	Roth.
3.26	4.02	60	1615	27.8	878	Higgins.
2.85	3.32	47	1348	23.3	733	Carpenter.
2.96	3.69	68	1472	25.5	800	Higgins.
3.37	4.06	72	1616	28.3	883	Carpenter.
3.29	3.85	61	1539	27.0	844	Roth.
3.42	3.79	66	1531	26.9	841	Higgins.
3.42	4.30	62	1687	29.7	927	Emmes.
—	3.84	59	1524	26.9	838	Smith.
3.59	4.30	63	1697	30.0	938	Carpenter.
3.54	4.12	64	1629	28.9	900	Smith.
3.37	3.96	60	1562	27.7	863	Smith.
3.19	3.90	51	1522	27.1	846	Carpenter.
2.86	3.50	58	1341	24.3	753	Higgins.
3.19	4.04	66	1545	27.9	862	Roth.
3.14	3.68	61	1421	25.8	798	Emmes.
3.46	4.23	68	1612	29.4	906	Carpenter.

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TABLE 1—Continued.

Metabolism of

SUBJECT	AGE	OBSERVATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL PER MIN.	
		Days	Periods			Carbon dioxide	Oxygen
	<i>years</i>			<i>kilos</i>	<i>cm.</i>	<i>cc.</i>	<i>cc.</i>
V. G.....	17	17	71	54.3	162	198	233
A. F. G.....	24	1	3	53.9	175	178	207
M. B.....	27	3	9	53.6	160	170	210
L. E. A.....	30	1	3	52.2	174	191	219
B. M. K.....	27	1	4	51.4	163	164	234
B. N. C.....	32	1	3	50.6	179	192	213
J. J. G.....	21	7	20	50.2	164	175	203
E. J. W.....	58	1	3	50.0	155	142	165
F. P.....	17	1	3	49.3	161	188	229
V. E. H.....	21	1	3	49.3	163	157	198
T. M. C.....	35	17	93	48.5	165	156	185
J. H.....	26	6	12	45.5	154	153	173

temperatures averaging above this will so affect metabolism as to preclude studies under those conditions. What is the normal pulse, and what is the normal temperature of any given subject will always be a question. Individuals who otherwise exhibit no abnormalities, having a pulse rate close to 100 per minute, or having a buccal temperature averaging 99.5° F. (37.50°C.) or above, must in all probability be excluded. The ideal condition therefore for studying the minimum, basal metabolism of a normal individual is during complete muscular repose, in the post-absorptive state, with the pulse rate well below 100 and with the buccal temperature not over 99.5° F. (37.50°C.)

While heretofore the relatively slight use of the few normal experiments reported by Zuntz and his coworkers has laid emphasis only upon fluctuations in body weight and the computation of the metabolism per square meter of body surface, it has become increasingly evident that factors which may no longer be neglected are height, age, and sex, and hence in this report these additional data are given specifically for discussion in a subsequent series of papers.

While we are able to present the average values from a large number of men and women, we recognize that the accumulation of

normal men.

PER KILO PER MIN.		PULSE RATE	HEAT PER 24 HRS. (CALCULATED)			OBSERVER
Carbon dioxide	Oxygen		Total	Per kilo of body weight	Per square meter of body surface	
cc.	cc.		cal.	cal.	cal.	
3.65	4.29	59	1632	30.1	922	Carpenter.
3.30	3.84	61	1453	27.0	826	Emmes.
3.17	3.92	61	1455	27.1	831	Carpenter.
3.66	4.20	67	1541	29.5	896	Roth.
3.19	4.55	75	1579	30.7	929	Emmes.
3.79	4.21	61	1510	29.8	893	Roth.
3.49	4.04	57	1425	28.4	848	Carpenter.
2.84	3.30	43	1158	23.2	693	Roth.
3.81	4.65	60	1591	32.3	958	Roth.
3.18	4.02	53	1365	27.7	822	Roth.
3.22	3.81	69	1292	26.6	788	Emmes.
3.37	3.80	68	1223	26.9	779	Smith.

normal data has only just begun, and while we recognize the certain number of variables which should be considered and will be considered in our subsequent discussion, such as height, age, weight and sex, it is not at all unlikely that with further study we may expect to find differences with occupation, character of the foregoing diet, general muscular development, and particularly general tone or condition of the body. It is thus clear that our data must be looked upon only as a skeleton, to which may be added other data for forming a more complete picture of the normal metabolism, or the variations that may be expected in the normal metabolism of men and women of varying height, weight, and age.

Methods of study. The larger number of experiments made and here reported were made with a universal respiration apparatus, devised in this laboratory.

In the earlier stage of the development of this respiration apparatus,⁶ to provide for the expansion of air in the closed air circuit, a rubber bathing cap was used. Subsequently this was replaced by a spirometer,⁷ to enable a better study of the mechanics of

⁶ The first description of this apparatus appeared in the *Amer. Journ. of Physiol.*, xxviii, p. 29, 1911.

⁷ The more recent and more perfected form is described in the *Deutsch. Arch. f. klin. Med.*, cvii, p. 157, 1912.

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TABLE 2.

Metabolism of

SUBJECT	AGE	OBSERVATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL PER MIN.	
		Days	Periods			Carbon dioxide	Oxygen
	<i>years</i>			<i>kilos</i>	<i>cm.</i>	<i>cc.</i>	<i>cc.</i>
Dr. M. D.....	44	1	2	93.6	165	202	256
Miss O. A.....	21	1	3	90.2	164	203	254
Miss H. H.....	21	2	6	88.3	161	190	228
Mrs. H. D.....	42	2	7	80.1	157	184	233
Miss C. Z.....	39	2	6	67.2	170	176	220
Miss S.....	27	2	5	65.5	171	178	202
Miss C. H.....	25	2	7	63.4	166	156	207
Miss A. K.....	23	2	6	63.2	171	165	202
Miss A. G.....	21	2	7	63.0	161	153	192
Miss V. A.....	21	2	6	62.9	168	167	188
Miss C.....	22	2	6	61.9	168	177	203
Miss K. K.....	21	1	4	61.5	154	—	238
Dr. A. B.....	32	1	4	60.3	163	197	207
Miss L. G.....	38	2	6	59.5	159	134	173
Miss B. W.....	22	2	6	59.4	162	180	223
Miss L. U.....	23	2	7	59.3	169	176	207
Miss M. W.....	25	2	8	58.6	167	168	206
Miss M. P.....	28	2	7	58.1	168	170	222
Mrs. E. B.....	53	1	3	58.0	163	171	202
Miss M. M.....	18	1	4	57.9	164	191	207
Miss E. P.....	23	2	6	57.7	175	166	207
Miss L. K.....	22	2	6	56.8	166	153	199
Miss E. A.....	15	2	5	56.8	157	205	231
Miss A. M.....	20	2	8	56.8	152	155	192
Miss J. C.....	22	2	6	55.1	162	157	198
Miss G. L.....	21	2	7	55.0?	166	173	214
Mrs. D. C.....	36	3	8	54.9	153	160	182
Miss M. T.....	20	2	7	54.5	164	147	196
Miss F. K.....	18	2	5	54.1	164	157	179
Miss J. N. B...	26	1	3	53.8	160	134	178
Miss L. T.....	31	2	8	53.6	155	152	178
Miss F. E.....	22	2	8	53.1	162	161	202
Miss L.....	25	2	6	52.4	168	162	188
Miss A. D.....	37	2	5	52.3	166	159	196
Miss B.....	21	2	6	52.2	158	173	202
Miss R. M.....	16	2	6	52.1	162	159	195
Miss L. J.....	24	1	3	51.8	159	142	179
Mrs. A.....	29	1	3	51.6	163	166	205

normal women.

PER KILO PER MIN.		PULSE RATE	HEAT PER 24 HRS. (CALCULATED)			OBSERVER
Carbon dioxide	Oxygen		Total	Per kilo of body weight	Per square meter of body surface	
<i>cc.</i>	<i>cc.</i>		<i>cals.</i>	<i>cals.</i>	<i>cals.</i>	
2.16	2.74	64	1765	18.9	695	Roth.
2.25	2.82	55	1756	19.5	708	Roth.
2.16	2.58	68	1584	18.0	649	Emmes.
2.29	2.91	65	1606	20.1	702	Emmes.
2.62	3.28	68	1521	22.7	747	Roth.
2.71	3.09	54	1426	21.8	713	Carpenter.
2.45	3.27	65	1413	22.3	722	Emmes.
2.62	3.20	62	1402	22.2	717	Emmes.
2.44	3.04	63	1324	21.1	679	Emmes.
2.66	2.99	62	1324	21.1	680	Emmes.
2.85	3.28	72	1427	23.1	732	Emmes.
—	3.87	73	1666	27.1	868	Emmes.
3.27	3.43	61	1486	24.6	786	Emmes.
2.25	2.91	60	1187	20.0	633	Emmes.
3.03	3.76	84	1546	26.1	827	Emmes.
2.96	3.50	55	1448	24.4	774	Emmes.
2.86	3.52	68	1429	24.4	768	Emmes.
2.92	3.82	66	1518	26.2	823	Emmes.
2.95	3.48	73	1415	24.4	769	Roth.
3.30	3.58	67	1475	25.4	801	Emmes.
2.87	3.59	73	1430	24.9	780	Emmes.
2.70	3.51	73	1365	24.1	750	Roth.
3.61	4.06	65	1630	28.7	896	Emmes.
2.73	3.39	54	1329	23.4	733	Emmes.
2.85	3.59	51	1363	24.8	764	Emmes.
3.15	3.89	76	1480	27.0	832	Emmes.
2.91	3.32	70	1278	23.4	720	Carpenter.
2.76	3.60	74	1359	25.0	770	Emmes.
2.89	3.31	67	1262	23.4	716	Carpenter.
2.49	3.31	78	1215	22.6	694	Roth.
2.84	3.33	64	1247	23.3	713	Emmes.
3.04	3.80	82	1391	26.2	799	Emmes.
3.09	3.59	69	1321	25.2	763	Emmes.
3.04	3.74	88	1355	26.0	788	Emmes.
3.31	3.88	78	1409	27.1	819	Emmes.
3.04	3.75	70	1353	26.0	787	Emmes.
2.74	3.46	52	1235	23.8	722	Emmes.
3.22	3.97	75	1421	27.5	831	Carpenter.

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TABLE 2—Continued.

Metabolism of

SUBJECT	AGE	OBSERVATIONS		BODY WEIGHT WITHOUT CLOTHING	HEIGHT	TOTAL PER MIN.	
		Days	Periods			Carbon dioxide	Oxygen
	<i>years</i>			<i>kilos</i>	<i>cm.</i>	<i>cc.</i>	<i>cc.</i>
Miss J. M.....	16	1	3	51.4	158	—	220
Miss J. B.....	27	2	6	51.1	163	146	183
Miss R. A.....	21	2	6	50.8	155	161	184
Miss M. C.....	16	2	6	50.6	162	145	185
Miss E. C.....	25	2	8	50.5	164	155	192
Miss I. B.....	18	2	5	50.1	166	160	173
Miss E. S.....	25	1	4	50.0	164	154	195
Miss C. B.....	24	2	6	49.8	162	168	205
Miss G. J.....	22	2	6	49.7	160	128	166
Miss D. W.....	19	3	8	49.4	160	153	187
Miss M. H.....	27	2	5	49.1	151	135	171
Miss C. L.....	21	1	2	49.1	151	153	195
Mrs. C. E.....	74	1	3	48.9	164	122	160
Miss V. M.....	24	1	3	48.9	162	149	198
Miss M. S.....	24	2	7	48.5	159	167	216
Miss G. F.....	27	2	6	48.5	155	140	180
Miss K. M.....	22	2	4	48.2	161	161	184
Miss L. B.....	27	1	3	47.0	167	136	169
Miss E. T.....	22	2	9	46.7	164	151	195
Miss H. T.....	25	2	6	45.0	159	156	203
Miss R. W.....	21	1	3	45.0?	153	141	186
Mrs. A. L.....	29	1	3	44.9	159	147	184
Miss M. J.....	27	1	3	44.8	157	137	172
Miss J.....	24	5	14	43.0	159	139	165
Miss A. C.....	38	2	6	42.6	165	138	168
Miss D. A.....	19	1	3	41.5	154	136	176
Miss E. W.....	24	2	6	40.5	157	153	183
Miss J. T.....	36	1	3	40.0	163	139	186
Mrs. S. C.....	52	2	5	37.4	155	122	140
Mrs. A. A.....	43	1	2	35.6	170	119	169

respiration. The rubber bathing cap has not been used in this apparatus for some time, and practically all forms are now fitted with the spirometer. A careful series of comparisons testing the apparatus in its earlier state has been made by Mr. T. M. Carpenter of the laboratory staff, and he assures us that the results obtained on the two different apparatus are identical, and therefore perfectly

normal women.

PER KILO PER MIN.		PULSE RATE	HEAT PER 24 HRS. (CALCULATED)			OBSERVER
Carbon dioxide	Oxygen		Total	Per kilo of body weight	Per square meter of body surface	
cc.	cc.		cal.	cal.	cal.	
—	4.28	77	1541	30.0	906	Emmes.
2.86	3.58	76	1265	24.8	746	Emmes.
3.18	3.62	77	1296	25.5	766	Emmes.
2.86	3.66	83	1273	25.2	756	Emmes.
3.07	3.80	71	1327	26.3	788	Emmes.
3.20	3.46	70	1235	24.7	737	Carpenter.
3.08	3.90	65	1345	26.9	806	Emmes.
3.38	4.16	75	1408	28.3	843	Emmes.
2.58	3.34	63	1139	23.0	684	Emmes.
3.10	3.79	61	1300	26.3	786	Emmes.
2.74	3.49	66	1178	24.0	712	Roth.
3.12	3.97	78	1341	27.3	813	Roth.
2.49	3.27	63	1095	22.4	663	Emmes.
3.05	4.05	70	1351	27.6	819	Emmes.
3.45	4.45	74	1480	30.5	905	Emmes.
2.88	3.70	70	1233	25.4	754	Emmes.
3.34	3.82	69	1294	26.9	794	Carpenter.
2.89	3.60	66	1168	24.9	730	Roth.
3.24	4.17	87	1336	28.6	838	Emmes.
3.47	4.51	80	1393	30.9	892	Emmes.
3.13	4.13	75	1273	28.3	816	Emmes.
3.27	4.10	66	1272	28.3	815	Roth.
3.06	3.84	58	1189	26.5	767	Roth.
3.25	3.85	66	1158	26.9	766	Carpenter.
3.24	3.94	69	1168	27.4	779	Emmes.
3.28	4.24	76	1207	29.1	816	Roth.
3.78	4.51	82	1275	31.5	878	Emmes.
3.48	4.65	65	1269	31.7	881	Roth.
3.25	3.75	65	1013	27.1	734	Carpenter.
3.34	4.75	72	1141	32.0	858	Carpenter

comparable. All of the respiration apparatus used in the Nutrition Laboratory and the apparatus used at Syracuse University was constructed and tested at the Nutrition Laboratory. The apparatus used at Battle Creek was built there and was subsequently inspected, tested, and approved by two of us (P. R. and F. G. B.).

Certain of the results obtained in this laboratory with the bed

calorimeter are perfectly comparable and are here included. This calorimeter has been described in detail elsewhere.⁸ The technique and the manipulation of both the respiration apparatus and the bed calorimeter have received extended discussion in various recent publications, and it is unnecessary to enter into further discussion here. All of the operators in this investigation acquired their technique personally in the Nutrition Laboratory, and hence we have not only uniformity of apparatus and method, but uniformity of technique.

Plan of experiments. A considerable number of the values included in our paper were found incidental to other researches. Consequently they were mostly with men and generally include many periods on a good many days. Recognizing the rapid accumulation of material with regard to normal men, it was necessary to make a special effort to secure information with regard to the metabolism of normal women. Consequently special researches were instituted to add to our list of men, by including athletes and vegetarians, and to secure a large number of women experiments, and this present collection of data is the result. The routine involved that the subjects come to the laboratory in the morning about 8 o'clock, in the post-absorptive state, *i.e.*, at least twelve hours after the last food, thereby eliminating the question of the ingestion of food. They then lay down upon a couch or bed and remained perfectly quiet usually for one-half hour prior to the first experiment, this quiet being controlled either by a bed with graphic registering devices, indicating the slightest alterations in the change of position of the center of gravity of the body, or by a chest pneumograph which likewise registered slight muscular motions.

Experiments were usually made in several periods of fifteen minutes, with interims of fifteen to twenty minutes. Finally to secure the most representative value possible experiments were always made two and frequently many more days with the same subject. The pulse was always taken and usually the temperature. By thus making experiments in the period of repose and in the post-absorptive state with normal temperature, the influence of

⁸ Benedict and Carpenter: *Respiration Calorimeters for Studying the Respiratory Exchange and Energy Transformations of Man*, Carnegie Institution of Washington, Publication No. 123, 1910.

muscular activity, fever and the ingestion of food was completely eliminated.

Factors recorded or determined. The factors recorded or determined were age, weight, height, sex, carbon-dioxide excretion, oxygen consumption, respiratory quotient, and in spirometer experiments, ventilation of the lungs, etc. To this were added the body temperature, taken in the mouth, with a mercurial clinical thermometer, and continuous records of pulse rate. A graphic registration of the degree of muscular repose accompanied every experiment. This was obtained by having the subject lie upon a bed, one side of which rested upon a knife edge, the other being supported by a spiral spring. The slightest change in the position of the level of the bed altered the tension on a pneumograph, which was connected with a tambour and kymograph.⁹ With the slightest motion of any kind, a disturbance of the regular straight line on the kymograph was noticed. We were thus able to dispense with ocular observation, and personal impression plays absolutely no rôle in the record of the degree of restlessness or repose of the subject. Only periods of complete repose were of value.

In the respiration calorimeter, obviously, since each experiment lasted one and one-half hours, not as great a degree of repose could usually be obtained as in the short fifteen minute periods on the respiration apparatus. On the other hand, the subjects were remarkably quiet, and being thoroughly enjoined as to the necessity for quiet did not exhibit any major extraneous muscular motion. While therefore one must assert that in the bed calorimeter the muscular activity was slightly above that on the respiration apparatus, nevertheless irregularities in the bed curves were usually so slight as to be entirely negligible. Furthermore in a number of other experiments made with men, comparing both the bed calorimeter and the respiration apparatus, periods of complete repose on both give essentially the same value.

Computations for basis of comparison. It is perfectly obvious that with a large man one would expect more heat to be given off,

⁹ Roth, in using the Battle Creek apparatus, has recently successfully substituted a rubber bulb (Politzer or ordinary syringe) for the pneumograph, with such success that the pneumograph has been discarded in the Nutrition Laboratory.

more carbon dioxide produced, and more oxygen consumed than with a small man. To secure some common basis for comparison has been the desire of physiologists for many years.

The metabolism per kilogram of body weight has been emphasized by a large number of writers, and, based upon Rubner's law that the heat production of living animals is proportional to the square surface, efforts have been made to compute the body surface, and from that the heat production per square meter of body surface. While by no means in harmony with the belief that the law of the square meter of body surface is of the significance that has been attributed to it, we recognize the general habit of thought of writers in this line, and hence have computed our values on the basis of square meter of body surface, and per kilogram of body weight. Usually the values per square meter of body surface correspond to the heat production per twenty-four hours, and the carbon dioxide and oxygen are expressed in cubic centimeters per kilogram of body weight per minute.

Obviously although several of these experiments were made with the bed calorimeter, the majority were made with the respiration apparatus, and hence we have computed indirectly the heat from the carbon dioxide and the oxygen. Indeed the values for heat directly measured with the bed calorimeter are purposely here omitted, as we are not discussing the question of direct versus indirect calorimetry at this time. The computation of the heat production is usually based upon the oxygen consumption, making allowances for the change in the calorific equivalent of oxygen with varying respiratory quotients. The calorific value of oxygen is much more nearly constant, irrespective of the character of the catabolism, than is the caloric value of carbon dioxide, and hence in practically all of the cases we have used the oxygen consumption. In a few instances where the oxygen determinations were faulty we have used the carbon-dioxide production. In all such instances, when either the oxygen determinations were missing or when the carbon-dioxide production was missing, we have assumed, when no better evidence is available, a common respiratory quotient of 0.85. In certain cases where we have on the day previously or the day subsequently well determined quotients we have used those. But usually the quotient of 0.85 is used.

As in these short experiments it was frequently difficult to secure

accurate collection of urine we have not attempted to compute the calories from protein nor the non-protein respiratory quotient, but have used the calorific equivalent of oxygen as used by Zuntz and Schumburg,¹⁰ making no special correction for the influence of the protein metabolism upon the respiratory quotient and the calorific equivalent of carbon dioxide and oxygen. In short experiments, particularly with uncertainty as to the nitrogen excretion in the urine, this procedure is recommended by Loewy¹¹ as giving results practically within 1 per cent. The results of our observations are given in tabular form herewith. It should be stated that these results are given with no effort at this time to use them in the discussion of several important questions in metabolism, which will be subsequently presented in forthcoming articles, such as comparisons of the metabolism of men and women, vegetarians and those living on a mixed diet, athletes and non-athletes, and the influence of varying body condition, age, height, and weight on metabolism.

These values were, as has already been stated, obtained on people in presumably good health. They include no obviously pathological cases and are sufficient in number to be fairly representative of the two sexes, with considerable range of weight, although admittedly deficient in people of middle and old age. These deficiencies will be supplied at the earliest opportunity and it is our purpose to revise the tables from time to time. People who are distinctly over- or underweight, provided no other reason for their exclusion was found, have purposely been included even at the risk of a misuse of the term "normal."

¹⁰ Zuntz and Schumburg: *Physiologie des Menschen*, Berlin, 1901, p. 361.

¹¹ Loewy: *Oppenheimer's Handbuch der Biochemie*, Jena, iv, p. 281, 1911.

THE PURINE ENZYMES OF THE ORANG-UTAN (*SIMIA SATYRUS*) AND CHIMPANZEE (*ANTHROPO-PITHECUS TROGLODYTES*.)

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(Received for publication, May 14, 1914.)

So many recent studies on the behavior of purines in the metabolism of different species of animals are in agreement in their main results, that it is safe to draw from them certain conclusions. One is that the way in which different species metabolize their purines, and also the tissues which are most actively concerned in purine metabolism in various species, show marked and characteristic differences. Another is that of all the mammals yet investigated, man and the chimpanzee alone seem unable to destroy the greater part of the uric acid formed in metabolism, and to excrete it as the more soluble allantoin. Third, we find that the action of tissue extracts upon purines *in vitro* under suitable conditions corresponds in most respects with the action of the living organism upon the same purines; for example, human tissues will not destroy uric acid *in vitro*, and the liver and spleen of swine are deficient in the guanine-deaminizing enzyme, corresponding with the known tendency for guanine to be deposited in the tissues of swine, causing "guanine gout."

A recapitulation of the evidence on the distribution of the various enzymes concerned with the catabolism of nucleic acid, as demonstrated by *in vitro* experiments, shows that *nuclease* seems to be present in all cells, that *adenase* is demonstrable in nearly all mammalian tissues except those of the adult human being,¹ and that *guanase* is also nearly universally distributed in

¹ Concerning the presence of adenase in the human fetus there is at present some doubt; see Long: this *Journal*, xv, p. 449, 1913.

cells, being missed from the spleen and liver of the pig, the pancreas of the dog, and the human spleen.² The oxidizing enzymes have a more limited distribution, being usually demonstrable in but certain tissues of the animals in which they are found, and most often present in the liver; for example, in man xanthine-oxidase is found only in the liver, and uricase has been found practically only in the liver and kidneys in any species.

As before stated, the human tissues have repeatedly been found to exert no uricolytic power whatever upon uric acid added to fresh tissue extracts or emulsions, which corresponds with the fact that man excretes much uric acid and only traces of allantoin, the latter probably coming chiefly if not entirely from the food. Even the monkey was found by one of us³ to have uricolytic enzymes in its liver, and Hunter and Givens⁴ found, in agreement with this, that the monkey excretes little uric acid, its place in the urine being taken by allantoin as with all the lower mammals. Also, in destroying adenine readily, the monkey seems to resemble the lower animals rather than adult man, who, as found by Jones and his coworkers, is unable to convert adenine into hypoxanthine *in vitro*.

On account of this discrepancy between man and monkeys, there is much significance in the observation of Wiechowski⁵ that the chimpanzee excretes only uric acid and no allantoin in its urine, thus resembling man and differing from all other mammals, as brought out especially well by the tables recently published by Hunter.⁶ In view of the great interest concerning the relationships between man, the anthropoids, and the monkeys, these new points of resemblance and difference have especial importance, and hence we have sought to extend our knowledge in this field by a study of the purine enzymes present and demonstrable in the tissues of the anthropoid apes. Fortunately, through the great kindness of Dr. W. T. Hornaday, director of the collection of the New York State Zoölogical Society, we came into

² These data are compiled, with bibliography, in this *Journal*, vii, p. 171, 1910.

³ Wells: this *Journal*, vii, p. 171, 1910.

⁴ This *Journal*, xiii, p. 371, 1912; xvii, p. 37, 1914.

⁵ *Prag. med. Wochenschr.*, xxxvii, p. 275, 1912.

⁶ This *Journal*, xvii, p. xxiii, 1914 (*Proc. Soc. Biol. Chem.*).

possession of the fresh body of an orang-utan and also of a chimpanzee in suitable condition for our studies.

The orang-utan was an adult female which had been in the possession of the New York State Zoölogical Society for six years. It died of tuberculosis, and was immediately packed in snow and sent to us by express, arriving in fine condition and with the tissues apparently as fresh as if the animal had been dead but a few hours. (Here it may be stated that numerous observations in this laboratory have shown that the purine enzymes persist in the tissues long after death, even when there is considerable post-mortem change or putrefaction). Autopsy showed that the animal still possessed a considerable amount of fat tissue, although suffering from extensive tuberculosis. This was most marked in the spleen, which weighed 800 grams and was mainly composed of large caseous areas, the process resembling that commonly seen in the spleen of tuberculous guinea pigs. The lungs showed extensive caseous tuberculosis, especially in the upper lobes, with some ulceration, although the process seemed to be of an acute type. There were a few tubercles in the kidneys and liver, the latter also exhibiting considerable fatty metamorphosis. The tissues were ground up, suspended in toluene water, added to various quantities of purines in solution, and allowed to act upon the purines under the conditions detailed below:

EXPERIMENT I. *Liver* tested for *uricase*. To 100 grams of liver tissue was added 0.201 gram uric acid dissolved with a minimum amount of NaOH, and kept at 38–40°C. for forty-three hours, with a current of moist toluene-saturated air running through the mixture. The material was then coagulated by heat, filtered, and from the filtrate we recovered 0.149 gram uric acid and 0.100 gram xanthine.⁷ The identity of these substances was established by the solubility and crystalline structure of the uric acid, which gave a positive murexide test; and the solubilities, negative murexide and positive Weidel test of the xanthine.

According to this experiment the orang-utan liver exhibits no *uricase* activity. Furthermore, it seems able to convert its own purines into xanthine, but not to oxidize the xanthine to uric acid; that is, it does not contain xanthine-oxidase.

EXPERIMENT II. *Liver*, tested for *xanthine-oxidase*. To 100 grams of liver tissue was added 0.201 gram xanthine, and kept forty-three hours with

⁷ In all these analyses the method of Kruger and Salomon was followed.

air current as in Experiment I. Recovered 0.244 gram xanthine, giving all the characteristic reactions and negative murexide test. This corroborates the previous demonstration of the absence of *xanthine-oxidase* in this liver.

EXPERIMENT III. *Viscera*, tested for *xanthine-oxidase*. A mixture of different tissues, weighing all told 90 grams, consisting of about equal parts of small intestine, heart muscle, lung; and in addition 15 grams of pancreas, 25 grams of spleen, and one adrenal, was added to 0.301 gram xanthine. The digestion was continued forty hours with an air current, as in Experiment I, at the end of which time there was recovered 0.256 gram xanthine. No positive murexide test could be obtained from the purines, showing the absence of *xanthine-oxidase* action in the tissues under the conditions of the experiment.

EXPERIMENT IV. *Kidney*, tested for *xanthine-oxidase*. 50 grams of kidney tissue acted on 0.2 gram xanthine as in the preceding experiments. There was recovered 0.199 gram xanthine, and no material giving the murexide test. Here again there is no demonstrable *xanthine-oxidase*.

EXPERIMENT V. *Liver*, tested for *adenase* and *guanase*. To 100 grams of liver we added 0.18 gram adenine, 0.24 gram guanine, and 0.044 gram hypoxanthine. The mixture was permitted to autolyze with toluene in a tightly stoppered bottle for thirteen days at 37°C. at the end of this time there was recovered 0.135 gram xanthine, 0.115 gram adenine, 0.192 gram guanine, 0.077 gram hypoxanthine. From these results it would seem that this liver does not contain either *guanase* or *adenase*, but it is apparently able to form xanthine from the purines of its own nucleic acids during autolysis. This is the only apparent explanation for the fact that so much xanthine is recovered with practically no loss of guanine. While the recovery of adenine is low, but 64 per cent, yet in experiments of this sort little less than a quantitative conversion of purines is of significance.

EXPERIMENT VI. *Mixed viscera*, tested for *adenase* and *guanase*. To a mixture of about equal parts of stomach, ileum, lung, and heart muscle, weighing together 90 grams and also one adrenal was added: guanine, 0.24 gram; adenine, 0.18 gram; hypoxanthine, 0.044 gram. After thirteen days' anaerobic digestion, as in experiment V, recovered: guanine, none; xanthine, 0.383 gram; adenine, 0.141 gram; hypoxanthine, 0.063 gram. Here we find *guanase* present, and apparently some xanthine has also come from the tissue purines. *Adenase* seems to be absent.

EXPERIMENT VII. *Kidney*, tested for *adenase* and *guanase*. To 50 grams of kidney were added 0.24 gram guanine, 0.18 gram adenine, 0.044 gram hypoxanthine. After thirteen days' anaerobic digestion recovered no guanine, 0.334 gram xanthine, and but 0.062 gram adenine and 0.074 gram hypoxanthine. This experiment shows the presence of *guanase*. Concerning the *adenase* we are in doubt. There has been a decided loss of adenine, but it was not all recovered as hypoxanthine. Whether this was ascribable to some analytical error or to a conversion of a part of the hypoxanthine into xanthine we have no way of determining. Of a total

of 0.224 gram adenine-hypoxanthine, we recovered but 0.136 gram. The work was conducted by the usual methods, and should have yielded all the adenine and hypoxanthine.

EXPERIMENT VIII. *Muscle*, tested for *adenase*. To 100 grams of thigh muscle we added 0.18 gram adenine and 0.044 gram hypoxanthine. After thirteen days' anaerobic digestion recovered 0.148 gram adenine and 0.110 gram hypoxanthine, no guanine or xanthine. Here *adenase* was apparently missing, the high recovery of hypoxanthine being ascribable to the normal presence of free hypoxanthine in muscle tissue.

EXPERIMENT IX. *Spleen* tested for *adenase*. The spleen was largely caseous, but 100 grams of it were added to 0.18 gram adenine and 0.044 gram hypoxanthine. After thirteen days autolysis we recovered but 0.080 gram adenine and 0.124 gram hypoxanthine.

EXPERIMENT X. *Pancreas* tested for *adenase*. To 15 grams pancreas was added 0.090 gram adenine and 0.022 gram hypoxanthine. After thirteen days' anaerobic digestion recovered 0.060 gram adenine and 0.032 gram hypoxanthine. Here, also, *adenase* was missing, or if present, was possibly destroyed by trypsin.

The chimpanzee was a male, apparently a young adult, shipped to us in ice and received in perfect condition. Autopsy showed the left lung entirely solidified by chronic caseous tuberculosis, and also the upper lobe of the right lung, the right middle and lower lobes being but little affected. The liver showed a little fatty change, but no tubercles. There were some tuberculous glands about the hilum of each lung. The spleen showed a very few nodular tubercles, and there were some ulcers in the small intestine. The kidneys were normal.

Purine enzymes were sought for according to the methods used with the orang-utan, with the following results:

Oxidizing enzymes, autolysis with air current forty-four hours.

1. *Liver*, 100 grams. Added 0.191 gram uric acid. Recovered 0.178 gram uric acid, 0.051 gram xanthine, and a trace of hypoxanthine. This shows the *absence of uricase*, and the formation of xanthine from the tissue purines.

2. *Liver*, 100 grams. Added 0.190 gram xanthine. Recovered 0.036 gram uric acid (crystals typical, murexide test positive); 0.186 gram xanthine (murexide test negative, Weidel test positive); 0.047 gram hypoxanthine. This demonstrates the presence of *xanthine-oxidase*, but evidently not active or not abundant in this specimen.

3. *Mixed viscera*. Consisted of 25 grams spleen, one testicle, one adrenal, and 90 grams of a mixture of about equal parts of intestine, lung, and

heart. Added 0.176 gram uric acid and 0.182 gram xanthine. Recovered 0.148 gram uric acid, 0.160 gram xanthine, and 0.038 gram hypoxanthine. Hence the mixed viscera gave no evidence of the presence of either *uricase* or *xanthine-oxidase*.

4. *Kidney*, 65 grams. Added 0.189 gram uric acid and 0.174 gram xanthine. Recovered 0.171 gram uric acid, 0.115 gram xanthine and 0.044 gram hypoxanthine. This demonstrates the absence of active *uricase* and *xanthine-oxidase* in the kidney.

ADENASE AND GUANASE EXPERIMENTS. Anaerobic autolysis for fourteen days, at 37°C.

1. *Liver*, 100 grams. Added 0.134 gram adenine and 0.133 gram guanine. Recovered no uric acid, no guanine, 0.156 gram xanthine, 0.073 gram adenine, 0.077 gram hypoxanthine. *Guanase* present, *adenase* probably absent.

2. *Kidney*, 65 grams. Added 0.134 gram adenine and 0.133 gram guanine. Recovered no uric acid, no guanine, 0.167 gram xanthine, 0.083 gram adenine, and 0.063 gram hypoxanthine. *Guanase* present, *adenase* probably absent.

3. *Spleen*, 75 grams. Added 0.134 gram adenine and 0.133 gram guanine. Recovered no uric acid, no guanine, 0.175 gram xanthine, 0.075 gram adenine, 0.078 gram hypoxanthine. *Guanase* present, *adenase* probably absent.

4. *Mixed viscera*, consisting of one testicle, one adrenal, and 30 grams each of lung, intestine, and heart muscle. Added adenine, 0.134 gram; guanine, 0.133 gram. Recovered no uric acid, no guanine, 0.163 gram xanthine, 0.087 gram adenine, and 0.055 gram hypoxanthine. *Guanase* present, *adenase* probably absent.

5. *Muscle (pectoral)*, 50 grams. Added 0.134 gram adenine and 0.133 gram guanine. Recovered no uric acid, 0.029 gram guanine (Weidel test negative); 0.105 gram xanthine, 0.064 gram adenine, 0.054 gram hypoxanthine. Apparently here there is but little active *guanase*.

6. *Pancreas*, 22 grams. Added 0.064 gram adenine and 0.073 gram guanine. Recovered no uric acid, 0.046 gram guanine, 0.056 gram xanthine, 0.053 gram adenine, and 0.046 gram hypoxanthine. Because the trypsin might have destroyed any purine enzymes present, it does not seem safe to conclude that adenase and guanase are actually missing, and from the partial conversion of the guanine into xanthine, it seems probable that guanase is present.

From the results obtained in these experiments it would seem that the orang-utan and the chimpanzee both resemble man in having no uricolytic enzyme demonstrable by *in vitro* experiments. This is in complete accord with the findings on the purine excretion of the chimpanzee, which according to Wiechowski, excretes much uric acid and no allantoin. We have, therefore, by these experiments, added another item of evidence as to the relationship of man and the anthropoids, to the effect that man and the anthropoids differ from all other mammals in their lacking the

uricolytic enzyme in their tissues. Hence, in this remarkable metabolic peculiarity, the anthropoids resemble man more closely than they resemble the monkey. This is also in accord with the biological serum reactions, for it has been shown that the blood of the anthropoids is more closely related to human blood in its precipitin reactions than to the blood of monkeys.

Apparently the adenase is missing from the tissues of the orang-utan, in which respect it also resembles man—at least adult man—and differs from the monkey. The relatively low recoveries of adenine in some of our analyses cannot be considered proof of the presence of adenase, for nothing less than an almost or entirely quantitative destruction or conversion is satisfactory evidence of the presence of purine enzymes. The same is probably true of the chimpanzee, there always being a large precipitate of adenine picrate recovered, which was typical as to its crystalline structure and its melting point, but which was always considerably less in amount than would correspond to the adenine which had been added. These results are unsatisfactory, and we have not been able to explain them; but we believe that we are safe in saying that adenase is missing from the tissues of both orang-utan and chimpanzee.

Guanase seems to be generally distributed in both animals, except in the liver of the orang-utan and possibly the muscles and pancreas of the chimpanzee. The lack of guanase in the orang-utan liver is not duplicated in man, in all of whose tissues except the spleen, guanase seems to be present. Also the absence of xanthine-oxidase in the orang-utan liver is a difference—in fact, it is difficult to explain this last feature, for all other mammals have been found to have xanthine oxidase in the liver or some other tissue. As will be seen, none of the usual purine enzymes was found in this orang-utan liver. It is, however, interesting to observe that the chimpanzee liver, although able to form uric acid from xanthine, is not very active in this respect. The deficiency of enzymes in the orang-utan liver can scarcely be ascribed to the fatty change that was present, since it has been shown by one of us⁸ that the maximum possible amount of fatty change in the liver of other animals causes no appreciable diminution in the

power of this tissue to oxidize uric acid and xanthine. Nor can post-mortem changes be held responsible, for these tissues were in good condition and we have found that liver can be kept in the ice box until putrid before any appreciable decrease in xanthine oxidase becomes apparent.

This is shown by the following experiments:

A dog's liver was ground fine, and kept in an ice box at 8-10°C. in an open porcelain dish. At the end of twenty-four hours, when there was no visible evidence of putrefaction, 50 grams were allowed to act for twenty-four hours with an air current in the usual way on 0.151 gram uric acid and 0.100 gram xanthine. All the purines present in this mixture were destroyed, so that no purine precipitate was obtainable.

After standing five days, when the liver had a sour odor, a 50-gram sample was digested with 0.153 gram uric acid and 0.102 gram xanthine. From this was recovered 0.044 gram uric acid, 0.076 gram xanthine, and a trace of hypoxanthine.

After standing seven days, when the tissue had become partly fluid, a 50-gram sample was digested with 0.150 gram uric acid, and 0.101 gram xanthine. Recovered 0.153 gram uric acid, the xanthine fraction being lost.

After nine days autolysis, when the liver was frothy and fluid, a 50-gram sample was digested with 0.151 gram uric acid and 0.103 gram xanthine. From the digest there was recovered 0.153 gram uric acid, 0.070 gram xanthine, 0.019 gram hypoxanthine, but no adenine.

Hence even after five days' standing in a disintegrated condition, the liver still destroyed a considerable proportion of the uric acid and xanthine added, there being recovered but 0.120 gram from a total of 0.255 gram that had been added, besides the considerable increment that must have come from the liver tissue itself.

SUMMARY.

The tissues of the orang-utan and chimpanzee were found to lack enzymes, demonstrable *in vitro*, which decompose uric acid in the presence of abundant oxygen. This finding agrees with Wiechowski's observation that the chimpanzee excretes uric acid and no allantoin in the urine. It also shows that the anthropoid apes resemble man in this respect, wherein they constitute a marked exception to all other mammals, including the monkey. *Man and the anthropoids are the only mammals that possess no demonstrable uricolytic enzymes.* As the presence or absence of the purine enzymes seems to be a developmental feature of differ-

ent species and classes of animals, this observation is a striking example of the close relationship of man and the anthropoids. Also, like adult man, the anthropoids seem to be defective or entirely lacking in adenase, but guanase is present in nearly all their tissues. Xanthine-oxidase seems to be present in the liver of the chimpanzee, as it is in man, but it was not demonstrated in any of the tissues of our specimen of orang-utan.

In closing we wish to express our indebtedness to Dr. W. T. Hornaday and the New York Zoölogical Society, who generously furnished the material which made this work possible.

THE SOLUBLE POLYSACCHARIDES OF LOWER FUNGI.

I. MYCODEXTRAN, A NEW POLYSACCHARIDE IN *PENICILLIUM EXPANSUM*.

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Much interest has been manifest among scientific investigators of recent years in the chemistry of lower fungi, chiefly because of the enormous importance of these organisms as disturbing factors in the preservation of foods and other agricultural products. The main interest has centered in their pathogenic properties. The study of these fungi has been chiefly from the standpoint of the mycologist and plant pathologist. It is only very recently that any extensive chemical investigations have been undertaken, and these have been confined, for the most part, to studies of the enzyme activities, nutrition, and toxic properties of the fungi.

In order to gain a true insight into the nature of the lower fungi and their effect upon their environment, a knowledge of their chemical composition is of fundamental importance. Proximate analysis shows the presence of carbohydrates, proteins, fats, and mineral elements, but furnishes no basis of differentiation of the fungi from the higher plants. It is obvious, therefore, that the various constituents must be isolated and studied individually. This paper presents a study of a soluble polysaccharide isolated from the apple fungus, *Penicillium expansum*, which has heretofore not been recognized in fungi, nor observed in any of the higher plants.

The organism, *Penicillium expansum*, was selected for these studies because of its importance in the apple industry and because of the readiness with which it can be propagated in artificial culture media and thus obtained in sufficient quantities for chemical investigation. Although it is widely distributed, it occurs characteristically upon decaying apples and other pomaceous fruits.¹

¹ Thom: Cultural Studies of Species of *Penicillium*, Bureau Animal Industry Bulletin, No. 118.

When mature, the cultures have a green or gray-green color, and generally emit a "fruity" odor. In synthetic media, such as Raulin's or Czapek's, it grows vigorously at room temperature. A full description of the morphology and habits of this organism may be found in Thom's paper.

HISTORICAL.

The carbohydrates of fungi have been studied in a number of instances but for the most part only by microscopic examination and qualitative chemical tests.

First of all, mention must be made of the fact that starch, which occurs in all chlorophyll-containing plants, is never present in fungi. Some statements are to be found in the literature with reference to the alleged occurrence of starch in fungi, but these have never been corroborated. Starch is said to occur in the spore tubes of some of the Ascomycetes,² in the sclerotium of ergot at the time of germination,³ in *Mycena tenerrima*,⁴ and in *Boletus pachypus*.⁵ In every case the only means of identification applied to the so-called starch were the iodine reaction and the digestion with diastase. As will be seen later, these tests alone are inconclusive. In ergot, the presence of starch may be readily accounted for by the probable contamination of the fungus substance with starch from the host plant.

Glycogen, otherwise known only as a product of animal metabolism, is widely distributed in the higher fungi and probably functions as a reserve substance in place of starch. Errera⁶ reports the occurrence of glycogen in *Peziza convexula*, *Acetabulum*, *Helvella esculenta*, *Morchella esculenta*, *Phycomyces nitens*, and other species, also in yeast and numerous Basidiomycetes. The identification of glycogen was, for the most part, microscopical. When glycogen is present, the cell contents have a viscid, highly refractive, opalescent appearance, and upon crushing the cell in water on a microscopic slide, the solution, after the addition of iodine, assumes a brownish-red color, which disappears on warming to 50-60° and reappears on cooling. Macroscopically, glycogen has been prepared by Reinke and Rodewald⁷ from *Aethalium septicum*, and by Clautriau⁸ from *Phallus impudicus*.

² Coemans: *Bull. soc. bot. de Belgique*, p. 78, 1862.

³ Belzung: *Recherches sur l'ergot de seigle*, p. 22, 1886.

⁴ Rolland: *Bull. soc. mycol. de France*, iii, p. 134, 1887.

⁵ Bourquelot: *ibid.* vii, p. 155, 1891.

⁶ Errera: *L'épithème des Ascomycètes et le glycogène des végétaux*, Thèse Bruxelles, 1892.

⁷ Reinke and Rodewald: *Studien über das Protoplasma*, Göttingen, 1881.

⁸ Clautriau: *Les réserves hydrocarbonées des Thallophtes*, 1889, p. 125.

Mycosulin was prepared by Biltz⁹ from *Elaphomyces granulatus*. He describes this as a fine granular powder, soluble in 240 parts of cold, and 5 parts of boiling water. Boiling with dilute sulphuric acid converted it into glucose. The substance was further investigated by Ludwig and Busse,¹⁰ who assigned to it the formula $C_{12}H_{22}O_{11} + H_2O$. The optical rotation was $+315^\circ$. They found that the substance was accompanied by another carbohydrate, mycodextrin, which had a rotation of $+120^\circ$.

Boudier¹¹ describes a pectin-like carbohydrate under the name of viscosin. This was prepared from the surface slime of several fungi, by dissolving in water and precipitating with alcohol. It was later found by Zellner¹² to be a complex mixture containing also nitrogenous substances and inorganic salts. One of the constituents was isolated by Winterstein¹³ and called paradextran. This was extracted from *Boletus edulis* by boiling with 2.5 per cent sulphuric acid and precipitating in the form of a jelly by adding alcohol. It was slowly soluble in 5 per cent potassium hydroxide, giving an opalescent solution. It had the empirical formula $C_6H_{10}O_5$, and, on hydrolysis, yielded glucose. On account of the opalescence of the solution, the optical rotation could not be determined.

By the same method Winterstein¹⁴ prepared para-isodextran from *Polyporus betulinus*. This likewise had the empirical formula $C_6H_{10}O_5$ and yielded glucose on hydrolysis. In 5 per cent alkali it showed a specific rotation of $+240^\circ$, and gave an intense blue color with iodine.¹⁵

From the foregoing it will be seen that our knowledge of the carbohydrates of fungi is based almost entirely upon investigations of the higher fungi. The molds have received very little attention in this regard, presumably because of the difficulty of obtaining material in sufficient quantities. Cramer¹⁶ obtained from the spores of *Penicillium glaucum* a carbohydrate which gave a flocculent precipitate with alcohol and a deep blue color with iodine. It hydrolyzed completely into glucose when boiled for three hours with 1.25 per cent sulphuric acid. It constituted about 17 per cent of the weight of the spores obtained from cultures of the organism on white bread. On account of the reaction with iodine, Cramer assigned the name "spore starch" to the substance. Its properties were not further

⁹ Biltz: *Trommsdorf's Journ.*, xi, p. 3, 1825.

¹⁰ Ludwig and Busse: *Arch. d. Pharm.*, cxxxix, 24, 1869.

¹¹ Boudier: *Die Pilze*, 1867, p. 48.

¹² Zellner: *Monatsh. f. Chem.*, 1906, p. 113.

¹³ Winterstein: *Zeitschr. f. physiol. Chem.*, xix, pp. 521-62, 1894.

¹⁴ Winterstein: *ibid.* xxi, pp. 134-151, 1895.

¹⁵ A number of other carbohydrate preparations from higher fungi have been described, but since the methods of purification employed are obviously inadequate, and the physical properties are not given in sufficient detail for identification, they will not be reviewed in this paper.

¹⁶ Cramer: *Arch. f. Hyg.*, xx, p. 197, 1894.

investigated. Alsberg and Black¹⁷ obtained a similar substance from *Penicillium puberulum*. The mycelium, which had been boiled, while fresh, with alcohol, was extracted with boiling water and filtered hot. The filtrate gave, on cooling, a small quantity of gummy material which iodine colored intensely violet, and which was not easily inverted by hot dilute hydrochloric acid. They considered this substance identical with the trehalum prepared from manna, and regarded it as the precursor of trehalose and mannitol.

EXPERIMENTAL.

Cultivation of Penicillium expansum.

Since considerable quantities of the fungus were required for this investigation, the organism had to be grown on rather a large scale. Cultivation in Petri dishes or flasks was too slow and laborious for the accumulation of sufficient material. It was found, however, that granite-ware pie pans with cake tins for covers, on the principle of the Petri dish, answered the purpose admirably. In this way a large surface was available for the growth of the mold, and since the covered pans could be stacked in the autoclave for sterilization, and subsequently in the cupboards after inoculation, a considerable saving in time and space was effected. The culture medium used was Raulin's fluid. This was poured into the pans to the depth of about 1 cm., the cover put in place, and the pans with their covers stacked in the autoclave and sterilized. After cooling, the contents were heavily inoculated with spores from pure cultures of *Penicillium expansum*. In about three days the surface of the liquid became covered with a dense mycelium, and the green spores appeared soon after. No difficulty was experienced in keeping the cultures free from contamination. After a week's growth the mycelium was removed, rinsed with water, and transferred to a Buchner press where it was subjected to a pressure of 350 kg. per sq. cm. It was then spread out to dry in the air over night, and finely ground in a mill. From fifty pans, about 20 cm. in diameter, the average yield was about 100 grams of the dry fungus.

¹⁷ Alsberg and Black: Bureau of Plant Industry Bulletin, No. 270, p. 34, 1913.

Extraction of the soluble polysaccharides.

The extraction was carried out on 100 gram lots of the finely ground, air-dried fungus, making two extractions with 700 cc. of distilled water each. The mixture was placed in a liter Erlenmeyer flask, left in a steam bath for two hours, and stirred frequently. The powder first swelled, and as the temperature rose there was a tendency to froth. After heating for some time the frothing ceased. The heating was continued for two hours, then the contents of the flask were strained through two thicknesses of cotton gauze while still hot. The residue on the cloth was subjected to a second extraction as before, and the extracts combined. The extracts, which still contained fine particles in suspension, were heated almost to the boiling point, and filtered on hot water funnels through pleated filters. A clear amber colored filtrate was obtained which, on cooling, became turbid and a white flocculent precipitate gradually settled out.

This precipitate was collected on a hardened filter, using a Buchner funnel and suction. A slimy white mass was thus obtained which gave an intense blue color with iodine. Since the clear filtrate also gave an intense iodine reaction, it was supposed that this might account for the reaction given by the white precipitate. This assumption proved to be correct. On redissolving the precipitate in hot water, cooling and filtering, and repeating this operation several times, the white pasty residue no longer gave a color with iodine. It was transferred to a beaker, stirred thoroughly with alcohol, filtered, washed a second time with alcohol, and finally with ether. The product was a fine snow-white powder. This was further dried *in vacuo* over sulphuric acid. The treatment with alcohol and ether is necessary in order to secure a fine powder which can be dissolved with ease. If the paste is dried directly, a hard horny mass results which goes into solution with difficulty.

The filtrate, obtained as above, gave an intense blue color with iodine. The polysaccharide to which this reaction is due is now being investigated and will be described in a later paper.

The clear amber filtrate from this second carbohydrate gave no iodine reaction, but gave a strong reduction of Fehling's solution. When evaporated on the steam bath, it left a brown gummy

residue with an odor resembling that of meat extract. After several days' standing, needle-shaped crystals separated out. These crystals, after purification by recrystallization from alcohol, melted at 166°. Since mannitol, which melts at this temperature, is known to occur in many fungi, there can be no doubt that these crystals were mannitol. Some white, spherical masses were also present which did not dissolve in alcohol. They did not melt at 230°, and on further heating they charred, leaving no ash. The nitrogen content was 10.94 per cent; theory for leucine, 10.69 per cent. The gummy mass gave a pink biuret reaction, a strong reduction of Fehling's solution, and left a considerable residue on ignition. It probably consisted of peptone, glucose, inorganic salts, and probably also amino acids, and other unidentified substances. Further investigation of this residue was not attempted.

The insoluble residue from the extraction of the fungus undoubtedly contains other carbohydrates not soluble in hot water. Pentosans¹⁸ have already been demonstrated in this and related species of molds. Hemicelluloses are probably present, also a chitin-like substance which on hydrolysis yields glucosamine. The exhausted residue, after drying, was set aside in the hope that opportunity will be afforded for further work on this problem at a future date.

The yield of the polysaccharide, prepared and purified as above, was about 2 per cent of the air-dry fungus. Cultures three days old and cultures seven days old showed no essential difference in the percentage yield. Qualitative tests showed the absence of nitrogen. On ignition, a 0.5 gram sample left a residue of 0.00045 gram; ash, 0.09 per cent. In all, about 40 grams of the substance were prepared.

Properties of Mycodextran.

Solubility. The dry powder, prepared as previously described, swells in cold water, and on warming dissolves to a clear solution. When the solution is cooled, the substance separates out again in a white flocculent mass. From hot solutions containing 1 per cent or more, a stiff white paste results on cooling. It is readily soluble in the cold in sodium hydroxide and in hydrochloric

¹⁸ Dox and Neidig: *This Journal*, ix, p. 267, 1911.

acid; insoluble in ammonium hydroxide, acetic acid, and sodium carbonate. From the solution in caustic soda it separates out when carbon dioxide is passed in, or when the alkali is neutralized with acid. The hot aqueous solution is not precipitated by tannic acid or lead acetate. Schweitzer's reagent and zinc chloride dissolve the substance with ease. Heating with glycerol to 180° gradually dissolves it to a clear yellow solution, from which the original substance may again be precipitated by dilution with water.

Specific rotation. Half a gram of the substance was suspended in 5 cc. of water and the volume made up to 50 cc. at 20° by the addition of $\frac{N}{16}$ NaOH. A clear solution resulted, which was then examined in a 1 dm. tube with the polariscope. The average of eight readings was +7.25° V.

$$[\alpha]_D^{20} = +251^\circ$$

A 0.25 per cent solution in hot water was examined in a 2 dm. jacketed polariscope tube. The average of five readings at a temperature of 73° was +3.625° V.

$$[\alpha]_D^{73} = +251^\circ$$

Hydrolysis. A 2-gram sample was hydrolyzed by boiling under a reflux condenser for ten hours with 300 cc. of 2 per cent HCl. A small amount of humus was formed. The filtrate was concentrated on a steam bath, cooled, nearly neutralized with NaOH and the volume made up to 250 cc. at 20°. Three aliquots of 25 cc. were used for determining the copper reduction by Allihn's method. The resulting cuprous oxide weighed 0.4333, 0.4339, and 0.4335; average 0.4336 gram corresponding to 0.204 gram glucose, or 2.043 gram glucose for the entire solution.

The same solution was polarized in a 2 dm. tube at 20°. The average of six readings was +2.495° V.

$$[\alpha]_D^{20} = +52.9^\circ$$

$$[\alpha]_D^{20} \text{ for glucose} = +52.7^\circ$$

The remainder of the solution was boiled with sodium acetate and phenylhydrazine hydrochloride. A yellow osazone separated from the hot solution. After two recrystallizations from 50 per cent alcohol and drying *in vacuo*, the osazone melted at 205–206°. Phenylglucosazone melts at 204–205°.

The sole product of hydrolysis was, therefore d-glucose, hence the name *mycodextran* is proposed for this polysaccharide.

Refractive index. This was determined by means of a Zeiss dipping refractometer on the 1 per cent solution in NaOH used for the optical rotation.

Solution	<i>n</i>
1 per cent in 0.09 N NaOH	1.33582
0.09 N NaOH	1.33451
Difference.....	0.00131

Ultimate analysis. A sample was oxidized in an ordinary combustion furnace with cupric oxide, in a current of air followed by a current of oxygen.

0.1 gram substance gave 0.1613 gram CO₂ and 0.0539 gram H₂O. From these figures the following percentages of carbon and hydrogen were calculated, on the ash-free basis:

	Found:	Calculated for C ₆ H ₁₀ O ₅ :
C.....	44.03	44.44
H.....	6.05	6.17

Mycodextran has, therefore, the same empirical formula as starch and cellulose.

Action of enzymes. To determine the effect of amylolytic enzymes upon mycodextran, the following experiments were performed. One-tenth gram of the enzyme to be tested was dissolved in 10 cc. of water and added to a paste prepared by dissolving 0.1 gram mycodextran in 10 cc. of hot water and cooling. 2 cc. of a 1 per cent tricresol solution were added as an antiseptic. The mixture was allowed to stand for three days at room temperature, then filtered in Gooch crucibles, and the insoluble residue weighed after drying to constant weight. The filtrates were tested for reducing sugars.

Enzyme	Mycodextran recovered gm.
Malt diastase (0.1 gram).....	0.1060
Taka diastase (0.1 gram).....	0.0967
Pancreatin (0.1 gram).....	0.1177
Saliva (10 cc.).....	0.0986
Control (10 cc. water).....	0.0989

The recovery of unchanged mycodextran after three days' digestion is practically quantitative. In the case of pancreatin, the high result is probably due to adsorption of the enzyme. The filtrates showed no reducing power, except in the case of taka diastase, and here the enzyme alone gave practically the same amount of reduction. It is safe to conclude, therefore, that the ordinary amylolytic enzymes are without effect upon mycodextran.

Mycodextran resembles, in some respects, the lichenin prepared from *Cetraria islandica* by Hönig and Schubert.¹⁹ Lichenin is soluble in hot water, insoluble in cold water, is not acted upon by the ordinary amylolytic enzymes, and upon acid hydrolysis yields glucose. It does not, however, rotate the plane of polarized light. Winterstein's para-isodextran,²⁰ on the other hand, has a specific rotation of $+240^\circ$, but it gives a blue color with iodine. Winterstein does not state whether para-isodextran is soluble in hot water, or hydrolyzed by enzymes. The properties of mycodextran, described in the foregoing pages, do not correspond with those of any known polysaccharide, and it is evident that the substance has not been prepared by previous investigators. Its function is, apparently, that of a reserve carbohydrate, which is stored up in the fungus until the sucrose of the medium has become exhausted. Attempts to prepare mycodextran from cultures that had autolyzed for six weeks were unsuccessful.

¹⁹ Hönig and Schubert: *Sitzungsber. d. Akad. Wissensch. z. Wien*, xcvi, p. 685, 1887.

²⁰ Winterstein: *loc. cit.*

THE CONTRIBUTION OF BACTERIA TO THE FECES AFTER FEEDING DIETS FREE FROM INDI- GESTIBLE COMPONENTS.¹

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It is today a recognized fact that a considerable part of the dry matter of the feces is composed of the bodies of bacteria, the greater number of which are dead. How large a contribution to the excrement is furnished by those microorganisms that have found their way into, or been developed within, the alimentary tract cannot readily be estimated. As might be expected from the varying nature of the diet of adults, carrying with it quite unlike amounts of indigestible matter, the admixture of bacterial bodies must be widely different under different régimes. When one reads, in the text-books, that probably about one-third of the dry substance in the stools of normal adults is bacteria, it must be borne in mind that the statistics reported by different investigators range from 9 to 42 per cent. It is even stated that in infants with pronounced constipation two-thirds of the dry substance of the feces has been found to consist of bacteria.

The discrepancies cited are probably due in part to the unsatisfactory technique which has been employed for the estimation of the quantity of fecal bacteria.² To completely separate a group of bacterial cells from fragments of food and alimentary detritus

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

² Cf. Schmidt and Strassburger: *Die Faeces des Menschen*, p. 281, 1905; Hecht: *Die Faeces des Säuglings und des Kindes*, p. 33, 1910.

by mechanical means, as is attempted in the current method of isolating and weighing the fecal bacteria by Strassburger's method or modifications thereof, approaches the impossible.

Feeding experiments which we have conducted for several years with albino rats, employing diets composed entirely of digestible nutrients—isolated protein, fat, starch, sugar, and "protein-free milk"—without any addendum like the resistant cellulose which enters into the usual dietary of the adult, have given an opportunity to study the fecal bacteria from a new angle. Microscopic examination of the feces from animals thus fed shows the prominent admixture of bacteria. It occurred to us that, assuming an adequate digestion of the insoluble components of the ration, the fecal residues of rats maintained on the diets consisting of isolated food substances might be demonstrated to consist largely, if not entirely, of bacterial masses, after suitable removal of other soluble ingredients. By ascertaining the amount of these residual masses the contribution of alimentary bacteria to the make-up of the feces might be more effectively demonstrated and measured.

Accordingly the feces obtained during a period of feeding with known intake were dried and ground. The slight admixture of hairs which were inevitably present was removed as far as possible by passing the dried feces through a sieve. The powder was then successively extracted with various reagents as follows:

Ether—to remove any remaining fats, fatty acids, and lipoids.

Absolute alcohol—which might remove residual compounds of a lipid character, bile constituents, soaps, etc.

80 per cent alcohol—to remove alcohol-soluble proteins, like zein or gliadin, which entered into some of the dietaries employed.

0.2 per cent hydrochloric acid solution—whereby inorganic salts, especially phosphates of calcium and magnesium, were removed.

Absolute alcohol containing 0.2 per cent HCl—to complete the decomposition and removal of calcium or magnesium soaps.

The *residue* was then analyzed for nitrogen and ash.

The *food mixtures* had a composition approximately as follows:

	Gm. per 100 gm. of food
Protein.....	18
Starch.....	26
"Protein-free milk".....	28
Lard.....	28

In the majority of the experiments the foods were essentially alike except in respect to the protein component. The data are summarized in the table.

An inspection of the figures shows that the so-called "utilization of protein," calculated in the conventional way, in line 6, is comparable with the average findings in recorded metabolism experiments on larger animals. It approximates 90 per cent in the case of the ordinary proteins (columns I to VIII). In the case of dietaries containing considerable of the alcohol-soluble protein zein, however, the loss of nitrogen by the feces is decidedly larger (columns IX to XVII).

It has long been recognized that estimations of protein utilization prepared on this basis fail to give any indication of the contribution of alimentary secretions, bacteria, etc., to the nitrogenous waste in the feces. Various methods have been proposed for ascertaining the true "metabolizable" nitrogen; *i.e.*, to make suitable correction for the fraction of the fecal nitrogen which cannot be charged to undigested or indigestible nitrogenous intake. How large the bacterial contribution to this may be is indicated by the figures for the "residue" from the feces, line 12, calculated from the weight of fecal solids which are not soluble in ether, alcohol, dilute acid, etc., and therefore are essentially free from fats, inorganic components, zein, etc.

The question will at once be raised as to the validity of referring to the "residue," line 12, as bacteria rather than undigested food residues or something else. In the first place the microscopic examination and staining properties of this residue at once suggest to the trained observer an almost uncontaminated mass of bacterial bodies. Secondly, and more convincing, are the data in regard to the composition of the "residue." When its nitrogen content is calculated *on an ash-free basis* (line 15), fairly concordant figures are obtained, ranging from 10.7 to 12.2 per cent. These correspond quite closely with numerous recorded analyses of nitrogen in bacterial cellular substances.³

If the foregoing conclusions are justified, it is apparent, from line 12, that a considerable part of the dry fecal matter is made up of bacterial bodies. It may well be that some of the ether-

³ Cf. Wheeler: *This Journal*, vi, p. 513, 1909.

	I	II	III	IV	V
Character of the protein in food.....	CASEIN	EDESTIN			LACT- ALBU- MIN
Rat No.....	396	124	394	415	528
Period of feeding.....					
1. Duration of experiment, <i>days</i>	80	21	7	21	49
2. Food intake, <i>grams</i>	755.0	175.0	61.8	174.0	433.1
3. Feces (dried), <i>grams</i>	38.3	8.4	3.7	12.2	25.4
4. N in food, <i>grams</i>	19.3	5.3	1.9	5.3	11.4
5. N in feces, <i>grams</i>	2.3	0.4	0.2	0.7	1.4
6. N utilization, <i>per cent</i>	87.8	92.5	90.4	87.4	87.5
7. Solids soluble in ether, <i>per cent</i>	6.4	6.7	11.4	13.3	7.3
8. Solids soluble in absolute alcohol, <i>per cent</i>	18.3	11.1	16.3	13.1	7.8
9. Solids soluble in 80 % alcohol, <i>per cent</i>		10.3		9.6	
10. Solids soluble in 0.2 % HCl, <i>per cent</i> ..	38.3	25.3	29.9	20.0	33.2
11. Solids soluble in absolute alcohol + 0.2 % HCl, <i>per cent</i>		5.7	6.4	5.4	17.3
12. Residue from feces, <i>per cent</i>	41.3	31.9	35.0	34.6	38.0
13. N in residue, <i>per cent</i>	9.9	11.4	11.2	11.1	10.6
14. Ash in residue, <i>per cent</i>	14.8	5.1	8.4	4.0	5.5
15. N in ash-free residue (calculated), <i>per cent</i>	11.6	12.0	12.2	11.6	11.2
16. Feces N in residue, <i>per cent</i>	65.5	72.9	76.2	70.7	70.6
17. Non-bacterial fecal N in terms of food N, <i>per cent</i>	4.2	2.0	2.3	3.7	3.7
18. Ash in feces, <i>per cent</i>	25.4	21.1	19.9	17.6	21.9

VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII
VETCH LEGUMIN		GLI- ADIN	ZEIN + CASEIN	ZEIN + EDES- TIN	ZEIN						
1104	1106	462	860	452 i	452 ii	892 i	892 ii	471 i	471 ii	471 iii	471 iv
63	49	21	56	14	70	28	11	21	32	35	31
269.0	279.0	198.0	530.0	111.0	481.0	139.0	44.0	172.6	281.6	268.4	229.7
15.6	15.8	11.7	57.5	5.0	33.1	10.7	5.0	20.7	30.2	30.4	23.7
8.1	8.4	6.0	13.4	3.1	13.9	3.4	1.1	4.3	6.8	6.5	5.6
0.8	0.9	0.6	5.1	0.4	2.0	1.0	0.5	2.1	2.4	2.3	1.9
89.9	88.7	90.7	61.9	85.8	85.6	71.1	51.2	48.4	64.5	63.8	67.0
7.0	6.4	10.4	3.8	6.4	7.1	6.1	4.5	5.2	5.6	6.8	5.5
8.0	7.5	7.9	30.1	24.0	14.7	21.1	21.1	62.6	19.5	13.6	39.6
5.0	5.4	12.4	22.5	21.0	12.4	33.6	31.4		26.6	26.5	
18.0	23.6	40.7	11.2		20.1	9.2	11.1		10.0	11.9	13.4
4.4	3.2		7.3	1.6	10.3	1.6	3.8	1.5	6.4	9.8	5.7
38.6	41.7	33.1	24.6	30.9	33.7	27.6	29.4	22.8	31.4	33.2	36.1
10.4	10.8	10.3	8.2	8.3	8.7	7.8	8.1	8.6	7.5	8.0	8.0
7.0	5.9	5.7	28.5	26.0	26.5	31.2	33.4	25.4	29.8	29.4	28.8
11.2	11.5	10.9	11.5	11.2	11.8	11.3	12.2	11.5	10.7	11.3	11.2
76.2	75.6	68.9	22.6	28.6	48.6	23.3	22.7	18.5	29.9	34.4	35.9
2.4	2.7	2.9	29.5	10.2	7.4	22.3	37.7	39.5	31.2	30.8	27.0
14.4	16.9	18.3	16.3	20.6	22.2	16.5		13.5	18.2	18.7	20.4

and alcohol-soluble, as well as acid-soluble components removed in the preliminary extraction processes belong, strictly speaking, to the bacterial cells; for many types of these organisms are known to be rich in ether-soluble compounds.⁴ If they could be estimated and included, the share of fecal residue due to bacteria would become even greater.

After allowance for the nitrogen represented by bacterial products, the residual nitrogen attributable to unutilized ingested protein is at best very small, amounting to 2 to 4.2 per cent (line 17, columns I to VIII). Only in the case of zein does the relatively poor utilization become apparent (columns IX to XVII, line 17). That this is due to unabsorbed zein becomes apparent in these cases from the large fraction of material soluble in alcohol (line 8)—a property characteristic of zein. In so far as the ordinary proteins, other than zein, are concerned, the digestion and utilization of protein is quite as perfect as is that of the fats and familiar carbohydrates (excluding cellulose), when allowance is made for the portion appropriated by bacteria in the alimentary tract.

Other data included in the tabular summary need not be discussed in detail. The point which we desire to emphasize is the availability of the method pursued for the indication of the extent to which bacterial cells may contribute to the make-up of the fecal residues.

⁴ Cf. the papers of Tamura: *Zeitschr. f. physiol. Chem.*, lxxxvii, p. 85, 1913; lxxxix, p. 289, 1914.

STUDIES IN CREATINE AND CREATININE METABOLISM

I. THE PREPARATION OF CREATINE AND CREATININE FROM URINE.

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As preliminary to a study of creatine and creatinine metabolism in the animal organism it was deemed desirable to find a simplified process for the preparation of perfectly pure creatine and creatinine.

The methods heretofore available for the preparation of these compounds have been practically only the procedures suggested by Folin.¹ For preliminary precipitation of the creatinine from the urine Folin adds picric acid, the creatinine being almost quantitatively precipitated as the double salt, potassium creatinine picrate. The precipitation of this salt under such conditions was first described by Jaffé,² and Folin first suggested its practical application to the preparation of creatinine from urine. There seems no question that picric acid is the best precipitant available for obtaining creatinine from urine, and, in the process described below, this precipitation is retained exactly as suggested by Folin. But in the further steps the procedure has been very materially changed. Folin adopted potassium bicarbonate (later the normal carbonate) as the substance with which the creatinine picrate was decomposed. In his first paper on the subject the decomposition was carried out at room temperature by rubbing the picrate and bicarbonate, together with a little water, in a large mortar for about an hour. In his later communication the decomposition was carried out at about 45°, where it proceeds much more rapidly.

¹ Folin: *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904, this *Journal*, xvii, p. 463, 1914.

² Jaffé: *Zeitschr. f. physiol. Chem.*, viii, p. 399, 1886.

There would be little object in giving here a detailed critique of the Folin process for preparation of creatinine zinc chloride and creatinine. The following are the chief reasons for seeking another procedure. The decomposition of the picrate by means of potassium bicarbonate or carbonate is unsatisfactory for several reasons. In the first place, it is slow and troublesome in the technique involved; secondly, there is a chance for conversion of considerable quantities of creatinine into creatine, which will be subsequently lost, and thirdly, the solubility of the bicarbonate and of the potassium picrate formed are great enough to interfere with obtaining a good yield of reasonably pure creatinine zinc chloride later on. In making use of the method for practical purposes we have found that the creatinine zinc chloride obtained is very impure, and that the yield is regularly low and uncertain. We have seldom recovered 40 per cent of the creatinine originally present in the urine as creatinine zinc chloride by Folin's process. While the method is not at all to be regarded as impractical, we have not found it very satisfactory where one desires to obtain considerable quantities of pure creatine and creatinine.

The following method has been in use in this laboratory for nearly three years, except as small modifications have been made to improve certain steps. The procedure is published now with a belief, founded upon a thorough trial, that where the directions are carefully followed the results will be uniform and satisfactory.³

Preparation of creatinine zinc chloride.

The urine (which must be undecomposed) is precipitated with picric acid as described by Folin. Eighteen grams of picric acid are used for each liter of urine,⁴ the acid being dissolved in boiling alcohol (approximately 100 cc. of alcohol for each 40 grams of picric acid) and this hot solution poured into the urine while the latter is thoroughly stirred. The mixture is allowed to stand over night and then the supernatant fluid is syphoned off. The residue

³ Myers and Fine (this *Journal*, xvi, p. 169, 1913) and Baumann (this *Journal*, xvii, p. 15, 1914) have reported satisfactory results, using the methods described in their paper.

⁴ It usually does not pay to work with less than 10 liters of urine at a time. We usually use 15 to 30 liters for each precipitation.

is poured upon a large Buchner funnel and drained with suction, after which it is washed once or twice with cold saturated picric acid, and sucked dry.

For decomposing the picrate, concentrated hydrochloric acid is used. This gives instantaneous and complete decomposition. The creatinine hydrochloride formed is very soluble in the strong acid, while the residual picric acid is nearly altogether insoluble.

The dry, or nearly dry picrate is treated in a large mortar or evaporating dish with enough concentrated hydrochloric acid to form a moderately thin paste (about 60 cc. of acid for each 100 grams of picrate is usually the right amount), and the mixture thoroughly stirred with a pestle for three to five minutes. The mixture is then filtered with suction on a *hardened* paper, the residue being washed twice with enough water to cover it, and sucked as nearly dry as possible each time.⁵ The filtrate is at once transferred to a large flask and neutralized with an excess of solid magnesium oxide.⁶ The oxide should be added in small portions, with cooling of the flask under running water between the additions. When all the hydrochloric acid has been neutralized the mixture will turn bright lemon yellow, or litmus paper may be used to test it. The mixture is then filtered with suction, the residue being washed twice with water. The filtrate is at once strongly acidified with a few cubic centimeters of glacial acetic acid and (paying no attention to a precipitate which may form at this point) the solution is diluted with about four volumes of 95 per cent alcohol,⁷ and filtered with suction any time after fifteen minutes from a slight precipitate (chiefly calcium sulphate) which forms. The final filtrate is treated with 30 per cent zinc chloride

⁵ Throughout this process the volumes should be kept as small as convenient, without involving too much loss through incompletely washed residues.

⁶ The commercial "heavy" variety is the best to use. Magnesium oxide is employed in place of any other alkali for two reasons, viz., (1) it is so insoluble that an excess will not convert creatinine to creatine, and (2) the chloride formed is so soluble both in alcohol and water as not to interfere in any later stage of the process.

⁷ In his later communications on the subject Folin does not add alcohol prior to precipitation of the zinc salt. It is used here for two reasons, viz., (1) a better yield is obtained than where it is not used and (2) the product is purer when precipitated from the alcoholic solution.

solution, using 3-4 cc. for each liter of urine originally used. This mixture is stirred (a precipitate should form almost immediately) and allowed to stand over night in a cool place.

The supernatant fluid is then poured off, and the precipitated creatinine zinc chloride collected on a Buchner funnel, washed once with water, then thoroughly with 50 per cent alcohol, finally with 95 per cent alcohol, and dried.

The product thus obtained should be a nearly white, light crystalline powder. The yield obtained depends, of course, mainly on the original creatinine content of the urine. Where care is exercised the above procedure will recover 90-95 per cent of the original creatinine, in the form of the zinc double salt, and thus the procedure is, when properly carried out, almost quantitative. Ordinarily, we obtain from 1.5-1.8 grams of creatinine zinc chloride per liter of urine.

Preparation of creatine from creatinine zinc chloride.

For the preparation of creatine the decomposition of creatinine zinc chloride by means of calcium hydrate has been adopted. When properly used this alkali will give a good conversion of creatinine to creatine with comparatively little loss during the process. It is possible that other procedures may be quite as good, but the following is comparatively simple and has been employed with good results for a long time. Following is the exact procedure. (The figures hold relatively for other quantities of the creatinine zinc chloride.)

One hundred grams of creatinine zinc chloride are treated with about 700 cc. of water in a large casserole and the mixture heated to boiling. One hundred and fifty grams of pure powdered calcium hydrate are then added, with stirring, and the mixture boiled gently for twenty minutes (with occasional stirring). The hot mixture is then filtered with suction, the residue being washed with hot water. The filtrate is treated with hydrogen sulphide gas for a few minutes and poured through a folded filter to remove the zinc. The filtrate is acidified by the addition of about 5 cc. of glacial acetic acid and boiled down rapidly to a volume of about 200 cc. This solution is allowed to stand over night, preferably in a cool place. The next day the crystallized creatine is filtered

off with suction, washed with a very little cold water, and then thoroughly washed with alcohol, and dried.⁸ This product is then recrystallized by dissolving in about seven times its weight of boiling water, and allowing the solution to cool slowly and stand for some hours. This product should be perfectly pure creatine. If necessary, it can be recrystallized again with very little loss. The crystallized product should be filtered off, washed with alcohol and ether, and dried in the air for about half an hour. Thus obtained, the creatine contains water of crystallization which it loses very readily upon exposure to air. To prepare creatine which can be weighed with absolute exactness, it is necessary to dehydrate this product by heating for some hours at about 95°.⁹

The yield in this process is about 18 grams of recrystallized creatine, and about 55 grams of creatinine zinc chloride recovered. Although over 50 per cent of the creatinine remains unconverted in this process, a longer boiling with the lime does not pay, since in twenty minutes a point is reached where creatine is decomposed almost exactly as fast as the creatinine is hydrolyzed to creatine. It is of interest to note in this connection that although a quantitative conversion of creatine to creatinine may be effected by chemical means, the reverse transformation has not, so far, been made to go to even approximate completion.

Preparation of creatinine from creatinine zinc chloride.

The preparation of absolutely pure creatinine has long been a difficult problem. The reasons for this lie chiefly in the facts that creatinine is strongly basic, retaining acids with great tenacity, and because in aqueous solutions it rapidly passes partially into creatine unless acid is present. It is questionable whether, prior to Folin's work upon the subject, even approximately pure creatinine had ever been prepared.

⁸ The filtrate obtained at this point should be diluted with alcohol and treated with zinc chloride (50 cc. of a 30 per cent solution) for recovery of unconverted creatinine.

⁹ In his most recent paper Folin advocates the use of creatinine zinc chloride as a standard for creatinine estimations in place of the potassium bichromate standard. Pure creatine prepared as described here may be employed as a satisfactory standard, after conversion into creatinine as described in the following paper. The residue of creatinine hydrochloride is permanent in solution.

During the present work three distinct procedures were developed which yielded samples of creatinine showing a theoretical nitrogen content, and reading slightly over 100 per cent by the colorimeter, using Folin's bichromate standard. The first two of these procedures have been practically discarded in favor of the third and simplest one, but one of them will be described because it may prove useful, and because of certain theoretical and practical points which it serves to bring out.

If creatinine zinc chloride be dissolved in its own weight of warm, concentrated hydrochloric acid and the solution diluted with several volumes of alcohol and allowed to stand some hours, preferably over night, the creatinine crystallizes out as a double salt of the formula $\text{Cr}_2\text{ZnCl}_2 \cdot 2\text{HCl}$. This salt has been described by Dessaignes¹⁰ but is mentioned in very few books describing creatinine and its salts. This hydrochloride of creatinine zinc chloride is insoluble in alcohol but very soluble in water. Upon slow evaporation of the latter solvent it can be obtained in very large beautiful white plates, but is more readily recrystallized by adding alcohol to the water solution. In the presence of a very great excess of hydrochloric acid in alcohol the hydrochloride of creatinine zinc chloride is decomposed or is not formed, the zinc chloride going into solution, while creatinine hydrochloride remains undissolved. This fact was taken advantage of in the following method for the preparation of creatinine.

The creatinine zinc chloride should be recrystallized before employing it for the preparation of creatinine, since this latter substance is not readily recrystallized.

For the recrystallization of the creatinine zinc chloride the following procedure has been adopted, though it involves a loss of about 10 per cent, owing to the use of animal charcoal. The figures hold relatively from other quantities. Ten grams of the creatinine zinc chloride are treated with 100 cc. of water, and about 60 cc. of normal sulphuric acid are added and the mixture heated to boiling until a clear solution is obtained. About 4 grams of purified animal charcoal are added, the boiling continued for about a minute, and then the mixture filtered with suction through a small Buchner funnel, the filtrate being poured back through the

¹⁰ Dessaignes: *Journ. de pharm. et de chim.*, 3, xxxii, p. 32.

same filter three or four times, until it runs through perfectly colorless. The residue is washed with hot water and the total filtrate is transferred to a beaker and while hot is treated with a little strong zinc chloride solution (3 cc.) and with about 7 grams of potassium acetate dissolved in a little water. After about ten minutes the mixture is diluted with an equal volume of alcohol and allowed to stand in a cold place for some hours and the crystallized product filtered off. The creatinine zinc chloride thus obtained contains a little potassium sulphate, which may be entirely removed by stirring it up with twice its own weight of water and filtering, washing with a little water, and then with alcohol. From 10 grams of the original salt, one obtains 8.5 to 9 grams of a pure snow-white preparation by this method.

The first method for the preparation of creatinine is as follows: Twenty-five grams of the recrystallized salt are treated in a flask with 100 cc. of alcohol which has previously been saturated with dry hydrochloric acid gas. The mixture is shaken well, warmed, then cooled and poured upon a hardened filter and filtered with suction. The residue is returned to the same flask and treated a second time in exactly the same way. This residue now contains very little zinc, and consists almost entirely of creatinine hydrochloride. It should be washed with ether and heated on a water bath until most of the excess of hydrochloric acid is removed. It is then dissolved in a little water and the solution diluted with four volumes of alcohol. Solid magnesium oxide is now added and the mixture stirred until blue litmus paper moistened with it and then with water, fails to turn red. This treatment removes all the hydrochloric acid from the creatinine. The mixture is filtered and concentrated by boiling, over a water bath, to about one-fourth of its volume, treated with a little ether, and allowed to cool. Creatinine crystallizes out, together with a very little magnesium chloride. The latter is removed by boiling the product once or twice with a little absolute alcohol. The residue, together with what crystallizes out from the absolute alcohol during the first hour, will consist of perfectly pure creatinine. Its solution in water should show no turbidity with silver nitrate, and should read at least 100 per cent colorimetrically, yield 4 to 6 grams.

The above process for the preparation of creatinine is not expeditious nor simple, nor is the yield very good. Below is detailed a

procedure which is interesting from the theoretical side, and which leaves nothing to be desired in simplicity or rapidity. An interesting point in connection with the process is that it was originally tried as an expeditious method for the preparation of pure creatine. Instead of yielding creatine, perfectly pure creatinine was obtained.

Following is the procedure. The recrystallized creatinine zinc chloride (which should be finely powdered) is placed in a dry flask and treated with seven times its weight (by volume) of concentrated aqueous ammonia. The mixture is slightly warmed and gently agitated by shaking until a clear solution is obtained, care being taken to drive off no more ammonia during the warming than is necessary to obtain a clear solution. The flask is stoppered, allowed to cool, and then placed in an ice box for an hour or more. Pure creatinine crystallizes out, and the first product obtained by this process should read 100 per cent colorimetrically. The yield is from 60 to 80 per cent of the theoretical. If the product is colored slightly yellow it may be recrystallized either from boiling alcohol, or by dissolving in five times its weight (by volume) of concentrated ammonia (warming enough to effect solution) and letting the solution stand in a cold place for some hours. Recrystallization is usually unnecessary. The product is perfectly pure, and may be used as a standard.

It is common knowledge that alkalies convert creatinine into creatine and the preparation of pure creatinine by crystallization from concentrated ammonia seems, upon theoretical grounds, an absurdity. As above stated, the process was first used in an attempt to prepare creatine. The explanation of the unexpected result obtained probably lies in the fact that such an excess of ammonia as is present in the concentrated solution acts as a sufficient dehydrating agent to prevent any hydration of the creatinine.¹¹ It may be added that creatine is not converted at all to creatinine by warming with concentrated ammonia. So it would appear that this latter solution is perhaps the only aqueous solution in which either creatine or creatinine may be dissolved without any change from the one into the other.

¹¹ Folin has called attention to the fact that ammonia developed in his autoclave process for converting creatine into creatinine does not interfere with the conversion under such conditions.

STUDIES IN CREATINE AND CREATININE METABOLISM.

II. THE ESTIMATION OF CREATINE.

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Folin proposed the estimation of creatine by conversion into creatinine, and the colorimetric estimation of this latter compound. For the conversion of creatine to creatinine Folin employed the procedure of heating the creatine solution with an equal volume of normal hydrochloric acid on the water bath for about three hours. Subsequently a modification of this procedure was suggested by Myers, who employed the autoclave and a higher temperature, by which means the time required for the conversion was shortened to about half an hour (exclusive of the time required for the heating and cooling of the autoclave). Rose later suggested the use of phosphoric acid in place of the hydrochloric acid in the autoclave process, this modification being applicable to sugar-containing urines. Very recently Folin¹ has suggested simple boiling and heating with picric acid to convert the creatine into creatinine, this procedure being applicable only to very small amounts of creatine.

Of the various procedures suggested it may be stated that the original Folin method yields wholly satisfactory results with normal urines, or with urines containing up to 5 per cent of glucose. The Myers modification is also satisfactory for normal urines, but gives results which are too high in presence of glucose, as pointed out by Rose. Glucose itself, however is not an interfering substance. The reason for the high results lies in the fact that under the conditions of the hydrolysis urea and sugar combine to form products which themselves give a coloration with picric acid in

¹ Folin: this *Journal*, XVIII, p. 469, 1914.

alkaline solution. Rose's phosphoric acid method is satisfactory only when carried out with the greatest exactness, and cannot be recommended as a general procedure. Folin's recent suggestion of the use of picric acid for the conversion of creatine into creatinine may be desirable under special conditions, but the fact that it requires some time to carry out, and especially that it is applicable only to very small quantities (about 1 mgm.) of creatine render it of less service for general work. To the mind of the writer the attempted replacement of all our standard methods of analysis by the so-called micro-methods lacks justification except when such methods must be employed from the necessities of the case. There is no question but that more certain and more accurate results are obtained where reasonably large quantities of a substance are taken for analysis.

A procedure for conversion of creatine into creatinine which the writer has found very satisfactory and expeditious consists in boiling the solution of creatine, to which has been added about an equal volume of normal hydrochloric acid, down to dryness. The procedure can be carried out in from five to ten minutes, and will (if the quantity of acid be somewhat increased) convert a gram or more of creatine in 20 cc. of water, quantitatively into creatinine. For either large or small amounts of creatine the conversion is absolutely quantitative.

When the procedure above suggested is applied to urine it is necessary to introduce a slight modification to prevent undue pigment formation. This modification consists in the addition, prior to the boiling, of a pinch or two of powdered or granulated lead. The lead is attacked only slightly by the acid, but the traces of hydrogen evolved serve to prevent oxidation of the pigments, and when urine is evaporated in this way and the residue taken up in water the solution will be found to have practically no more color than the original urine. This minimal pigment formation is a marked aid to a subsequent satisfactory color comparison.

The procedure for the determination of creatine in urine is as follows. Such a volume of urine as will contain between 7 and 12 mgm. of total creatinine is introduced into a small flask or beaker and from 10 to 20 cc. of normal hydrochloric acid are added, together with a pinch or two of powdered or granulated

lead. The mixture is boiled over a free flame as slowly or as rapidly as may be desired until very nearly down to dryness, when the heating should be continued to dryness either on a water bath, or very easily by simply holding the vessel in the hand and heating carefully for a moment or two. The residue should best stand on a water bath for a few minutes until most of the excess of hydrochloric acid gas has been expelled, after which it is dissolved in about 10 cc. of hot water and the solution rinsed quantitatively through a plug of cotton or glass wool (to remove all metallic lead) into a 500-cc. volumetric flask. 20-25 cc. of saturated picric acid are added, and about 7-8 cc. of 10 per cent sodium hydroxide solution, which contains 5 per cent of Rochelle salt. (The Rochelle salt should be present to prevent any formation of turbidity, which otherwise may occur, due to the presence of traces of dissolved lead. The tartrate has no effect whatever upon creatinine readings.) The flask is filled to the mark at the end of five minutes and read as usual.

The minimal pigment formation which occurs during the above described method offers distinct advantages over the original Folin procedure, or the Myers modification. When urines contain the usual quantities of creatinine the pigment formation does not affect the readings in the Folin or the Myers procedure, but when, as in children's urines, the total creatinine may be low, so that a large volume must be taken for the analysis and less diluted for the final reading, pigment formation becomes a factor of great importance. The new procedure is by far the most expeditious method as yet suggested for creatine conversion.

When the process is applied to pure creatine solutions or to beef extracts, or other solution where there is little development of color, the use of the lead may be omitted if desired. The residue in the beaker may then be treated directly with water, picric acid and alkali, and at the end of five minutes washed into a volumetric flask, diluted and read. An entire creatine estimation may be finished in this way in ten minutes.

As regards the accuracy of the process, it may be stated that it has been tested with pure creatine solutions of widely varying concentration (from 1 mgm. to 1 gram in 20 cc.), with normal and creatine-containing urines (human and dog), with urines to which known amounts of creatine have been added, and with beef and

muscle extracts. The results have been invariably within the limits of accuracy of the colorimeter. The method has been in constant use for three years in this laboratory, and has given continuous satisfaction. It is not, however applicable to urines containing glucose. For diabetic urines the original Folin procedure is probably the most satisfactory.

The explanation of the very rapid quantitative conversion of creatine into creatinine by evaporation with hydrochloric acid, as opposed to the very slow conversion when the mixture is simply heated, probably lies in the fact that the dry hydrochloric acid gas evolved at the end of the process is a very effective dehydrating agent. Since hydrochloric acid forms a constant boiling mixture with water it is immaterial as to just what quantity of hydrochloric acid is taken to begin with. The concentration of acid will always be the same toward the end of the process. However great the volume of solution taken to begin with, 20 cc. of normal hydrochloric acid will be sufficient, unless the quantity of creatine present exceeds 100 mgm.

So far as the writer is aware, this new procedure is the only one so far proposed which will give a quantitative conversion of creatine in relatively concentrated solutions. Of course the creatinine formed will exist in the residue as the hydrochloride. Starting with pure creatine, this method gives an excellent procedure for the preparation of standard creatinine solutions, where one prefers to use these in place of potassium bichromate.

STUDIES IN CREATINE AND CREATININE METABOLISM.

III. ON THE ORIGIN OF URINARY CREATINE.

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The question of the origin, the rôle and the fate of creatine and creatinine in the animal organism is one which is justly receiving much attention. Ever since Folin took up this question, and in mastering the analytical problems involved gave us one of the most beautiful processes known to analytical chemistry, the creatine-creatinine question has been prominent among the problems in metabolism. The questions are far from solved at the present time, but numerous recent researches are contributing to the elucidation of the subject.¹

For the past two years we have been studying the creatine-creatinine question in this laboratory in several of its phases, and in subsequent communications we shall discuss the questions of the interrelationship and fate of creatine and creatinine in the organism. The present paper is concerned primarily with the question of the origin of urinary creatine.

F. G. Benedict² first reported the elimination of creatine in the urine of starving men. Cathcart³ observed this fact independently, and since that time it has been repeatedly verified for human beings, and for the dog and the rabbit. Benedict originally

¹ For recent summaries of the literature on creatine and creatinine metabolism the reader is referred to the paper of Riesser: *Zeitschr. f. physiol. Chem.*, lxxxvi, p. 415, 1913; and to the papers of Myers and Fine; this *Journal*, xiv, p. 9, 1913; xv, pp. 283, 285, 1913; xvi, p. 169, 1913; Folin and collaborators: *ibid.*, xvii, p. 463, 1914.

² Benedict: Carnegie Institution of Washington, Publication No. 77, p. 386, 1907.

³ Cathcart: *Journ. of Physiol.*, xxxv., p. 500, 1907.

offered the hypothesis that the creatine appearing in the urine during inanition is derived from the muscle creatine. Dorner,⁴ Mendel and Rose⁵ and Howe and Hawk⁶ have offered data bearing upon this point, and finally Myers and Fine⁷ have presented experiments from which they conclude that "the creatine appearing in the urine during starvation is derived from the muscle tissue." Since Myers and Fine are the first to draw a definite conclusion as to the origin of urinary creatine, and since their paper gives an excellent summary of work upon this subject, as well as many interesting original data, we shall discuss their work in some detail after presenting our own results which bear upon this question.

The experiments which we wish to report were carried out upon phlorhizinized dogs. Cathcart⁸ first suggested a relationship between carbohydrate utilization and the appearance of creatine in the urine, as a result of noting that the creatine disappeared from the urine of fasting men upon giving carbohydrate food, while it did not disappear after administration of protein or of fat. Mendel and Rose developed the idea of a possible relationship between carbohydrate and creatine utilization in considerable detail, most of their observations having been made upon fasting rabbits. Wolf⁹ has pointed out that while giving protein to fasting rabbits may not affect the creatine output (as observed by Mendel and Rose) this observation does not hold for dogs, where the administration of protein, or of protein and fat is promptly followed by a disappearance of creatine from the urine. We have been able to confirm Wolf's observation in this connection. The fact that protein may be effective in causing a cessation of creatine elimination in the normal fasting dog need not, it seems to us, contradict the view of Cathcart and of Mendel and Rose that creatine metabolism is closely related to carbohydrate combustion, since Lusk has shown that in the normal dog a large proportion of

⁴ Dorner: *Zeitschr. f. physiol. Chem.*, lii, p. 225, 1907.

⁵ Mendel and Rose: *this Journal*, x, p. 213, 1911.

⁶ Howe and Hawk: *Journ. Amer. Chem. Soc.*, xxxiii, p. 215, 1911; Howe, Mattill and Hawk: *this Journal*, x, p. 417, 1911; Biddle and Howe: *Biochem. Bull.*, ii, p. 386, 1913.

⁷ Myers and Fine: *this Journal*, xv, p. 283, 1913.

⁸ Cathcart: *loc. cit.*

⁹ Wolf: *this Journal*, x, p. 473, 1912.

the protein ingested is converted almost immediately into glucose. Myers and Fine have recently suggested that the reason carbohydrate is effective in causing cessation of creatine elimination is because of its sparing action upon protein metabolism. Such an explanation would be more satisfactory were it not that the creatine *wholly* disappears upon a pure carbohydrate diet, while only a portion of the muscle tissue can be spared by such a diet. The experiments which we offer below also seem to call into question the adequacy of such a simple explanation.

As a result of work carried out upon incompletely phlorhizinized dogs Cathcart and Taylor¹⁰ concluded that the processes leading to acidosis and to creatine elimination are entirely distinct. Wolf and Osterberg reached this same conclusion as a result of work upon completely phlorhizinized dogs, and showed that such animals, when completely fasting, eliminate very large amounts of creatine. This was in line with the well known fact that the protein metabolism of fasting dogs under the influence of phlorhizin is increased 400-500 per cent over the simple fasting level, though Wolf and Osterberg pointed out that in one instance at least, the creatine elimination relatively outstripped the total nitrogen output.

Our experiments were planned to determine the effect of a creatine-free protein diet upon the elimination of creatine in dogs completely under the influence of phlorhizin. In the phlorhizinized dog we have a total non-utilization of carbohydrate, accompanied by a relatively enormous elimination of nitrogen and of creatine from the tissues. The large amount of nitrogen lost by the tissues may be partly, or as we have found in certain instances, completely, replaced or spared by exogenous protein, so that little or no extra destruction of nitrogenous tissue is taking place in the organism. But as Lusk and his collaborators have shown, the feeding of protein does not cause any utilization of glucose in the completely phlorhizinized dog. Hence we should have here a definite means of determining the origin or significance of the urinary creatine. If the creatine of the urine has its origin in the destruction of muscular tissue as has been assumed, a sparing of the body tissue destroyed by feeding exogenous protein should cause a corresponding fall in the creatine eliminated, whereas if the

¹⁰ Cathcart and Taylor: *Journ. of Physiol.*, xli, p. 276, 1910.

utilization (or destruction) of creatine be dependent upon carbohydrate utilization, the ingestion of exogenous protein in the phlorhizinized dog should not appreciably affect the output of creatine in the urine. If the feeding of protein has no influence whatever upon creatine elimination, we may conclude that creatine is constantly being formed in relatively large amounts in the organism, and that its proper handling probably requires (as one factor at least) the utilization of carbohydrate.

In the experiments detailed below the routine procedure was to fast the animal for two or three days, then inject phlorhizin daily, and feed creatine-free protein during definite periods. The phlorhizin was given subcutaneously once daily in 1.2-gram doses, suspended in 10 cc. of olive oil, according to the method of Coolen.¹¹ The protein used for feeding consisted either of washed blood fibrin (where the animals would take this diet) or of lean beef heart which had been finely ground and boiled for one or two days with successive changes of water until the final water and the pressed juice from the meat failed to yield the slightest reaction for creatine. Total nitrogen was determined by Kjeldahl's method, creatine and creatinine by Folin's methods (which we have found to be perfectly reliable in the presence of sugar and of the quantity of phlorhizin which we were using,¹² glucose by the titration method of Benedict.

Experiment I is detailed in Table I. The animal, a small black bitch with an initial weight of 7.5 kilograms, was completely under the influence of phlorhizin for eighteen days, after a preliminary fasting period of two days. This dog would eat washed blood fibrin in large amounts, so that it was possible, during three days at least, to keep the animal in a plus nitrogen balance in spite of the fact that there was practically no utilization of carbohydrate in the organism. No account is taken of the output of nitrogen through the feces, because, with the concentrated diets given in these experiments, the intestinal output of nitrogen was in many instances entirely negligible. Thus, the dog in experiment I

¹¹ Coolen: *Arch. de pharmacodynamic*, 1, p. 267.

¹² Wolf and Osterberg (*loc. cit.*) have previously reported that phlorhizin does not vitiate the Folin method for creatinine. The quantities of acetone bodies present in the urines with which we dealt in these experiments were found not to influence the results.

TABLE I.
Dog *sl.*

DATE 1913	WEIGHT kilos	URINE*							N IN FOOD grams	PHLORENZIN INJECTED grams
		Volume cc.	Specific gravity	Total N grams	Creatinine N gram	Body N lost grams	Creatino N gram	Creatinine gram		
March 23	7.50	60	1038	1.898	0.077	1.898		0.206	Fasting	0
March 24	7.38	58	1038	1.743	0.076	1.743		0.205	Fasting	0
March 25	7.12	315	1039	4.237	0.075	4.237		0.203	Fasting	1.2
March 26	7.00	520	1033	7.115	0.074	7.115		0.198	Fasting	1.2
March 27	6.70	825	1027	8.755	0.072	8.755		0.194	Fasting	1.2
March 28	6.50	930	1026	8.755	0.068	8.755		0.183	Fasting	1.2
March 29	6.16	2275	1011	10.880	0.071	6.980		0.191	3.9	1.2
March 30	6.06	2455	1010	11.842	0.067	3.142		0.180	8.7	1.2
March 31	5.98	2980	1009	15.031	0.066	0.781		0.178	14.25	1.2
April 1	6.00	2830	1009	12.570	0.062	0.970		0.167	11.60	1.2
April 2	6.02	2300	1011	13.500	0.059	1.440		0.159	12.06	1.2
April 3	5.70	1920	1008	6.558	0.053	6.558		0.143	Fasting	1.2
April 4	5.50	1800	1007	6.050	0.051	6.050		0.136	Fasting	1.2
April 5	5.30	1660	1007	5.343	0.046	5.343		0.125	Fasting	1.2
April 6	5.36	1700	1013	10.460	0.043			0.116	14.48	1.2
April 7	5.24	1790	1013	13.270	0.041			0.110	14.00	1.2
April 8	5.50	1240	1014	9.365	0.044			0.118	12.5	1.2
April 9	5.30	1660	1009	8.323	0.041	5.573		0.110	2.75	1.2
April 10	5.04	1630	1007	5.342	0.042	5.342		0.112	Fasting	1.2
April 11	4.96	1390	1008	6.390	0.042			0.113	6.6	1.2

* The urino was acid throughout.

had no feces for four days after feeding, when an elimination took place through the intestine which contained a total of 0.2 gram of nitrogen. A similar result was obtained in all the experiments except where the feeding was continued for some time. In these latter cases the nitrogen of the feces has been taken into account in the column under "Body N Lost."

During the first four days of the phlorhizin administration dog 31 received no food. The total nitrogen elimination during this period rose from the initial fasting level of 1.8 grams to 8.7 grams. The creatine elimination rose from zero to 0.727 gram, the quantity eliminated on the fourth day being four times the quantity of creatinine put out, the output of this latter substance having fallen from 0.205 to 0.183 gram, simultaneously with a rapid fall in body weight. On March 29, the fifth day of the phlorhizin injections, the dog was fed 3.9 grams of nitrogen in the form of washed blood fibrin. The total nitrogen output for this day was 10.8 grams, representing a loss of body nitrogen of 6.9 grams. Although this is nearly 2 grams less of body nitrogen loss than on the preceding day, the creatine has risen for this period to 0.757 gram, the largest creatine elimination recorded during the experiment. From this point the creatine elimination steadily falls, and it may be noted that this is the experience with phlorhizinized dogs which are fasting, or which are fed so little as to be rapidly losing body tissue. In later experiments, where the animals were fed continuously with a nearly adequate supply of food (from the nitrogen standpoint) it will be observed that the creatine elimination is nearly constant for a series of days (Tables V and VI.)

The point which is strikingly brought out in the experiment is that the creatine elimination is totally unaffected whether the nitrogen of the urine represents body tissue catabolized, or whether it is derived from exogenous creatine-free protein. The dog was fed March 30, 31, April 1 and 2. During these four days the dog lost a total of body nitrogen of only 5.8 grams, yet the creatine output for the same period amounts to 1.68 grams. During starvation, when the dog lost 8.7 grams of body nitrogen in one day, the creatine output amounted to 0.627 gram. The daily output of creatine during the feeding period was falling steadily, but we note that it continues to fall, and falls even more rapidly during the three following fasting days. During April 6, 7, and 8

the dog was in a plus nitrogen balance, yet the creatine output remains high and does not fall quite as rapidly as it did during the three previous fasting days, indeed for the first day of this period the creatine output rose from 0.182 gram for the previous fasting day to 0.196 gram. The latter day there was no loss of body nitrogen, but on the contrary a *retention of 4 grams*, while on the day previous a body loss of over 5 grams of nitrogen occurred. The slight rise in creatine elimination was not due to the feeding, for it sometimes happens during starvation, as can be seen in the similar rise in the creatine output on April 10. We think that an inspection of Table I will make it perfectly plain that there is no parallelism between body tissue destroyed, and creatine elimination. Sparing the body nitrogen does not affect the creatine output.

The creatinine elimination in experiment I is also of interest. It is apparently as independent of the creatine output as could well be imagined. With the exception of only two days, the creatinine eliminated steadily falls. At the end of the experiment the creatinine elimination is 45 per cent less than at the beginning, and the animal has lost 35 per cent of its initial body weight. Myers and Fine have explained the fall in urinary creatinine of fasting rabbits as due to a depletion of the body creatine, this latter substance being regarded as the direct source of the creatinine. That the rapid fall in creatinine recorded in the above experiment is due primarily to loss of living tissue, and not to a loss of initial creatine is shown in experiments V and VI, where the loss in body weight was much less, and the creatinine dropped very little, in spite of a long continued high creatine excretion.

In looking over Table I it is hard to imagine any appreciable conversion of creatine into creatinine in the organism when the relative amounts of the two substances eliminated undergo such enormous variation. It may be added that experiment I was discontinued with the animal still in good condition, except for the marked loss in body weight. Table III records another experiment with this animal.

Table II gives a summary of experiment II. Creatine-free meat was fed to the animal on the sixth and seventh days of the phlorhizin administration. Although it spared most of the body nitrogen, it will be noted that the creatine elimination was not notably affected, the quantity of creatine put out being apparently

Origin of Urinary Creatine

TABLE II.
Dog 33.

DATE 1913	WEIGHT		URINE*										N IN FOOD	PHILORHIZIN INJECTED
	Volume	Specific gravity	Total N	Creatinine N	Body N lost	Creatine N	Creatinine	Creatine	D:N	grams				
											grams			
May 3	kilos	cc.	grams	gram	grams	gram	gram	gram	gram		grams	grams		
May 3	13.04	166	3.670	0.152	3.670	0.019	0.409	0.059			Fasting	0		
May 4	12.88	135	3.134	0.139	3.134	0.015	0.373	0.046			Fasting	0		
May 5	12.68	120	3.552	0.142	3.552	0.024	0.383	0.074			Fasting	0		
May 6	12.16		8.032	0.147	8.032	0.160	0.397	0.500	4.0		Fasting	1.5		
May 7	11.84	600	11.385	0.138	11.385	0.292	0.372	0.910	3.0		Fasting	1.5		
May 8	11.44	660	9.440	0.127	9.440	0.286	0.341	0.891	3.4		Fasting	1.5		
May 9	11.26	960	13.823	0.116	0.793	0.191	0.313	0.596	3.7		Fasting	1.5		
May 10	11.24	800	13.755	0.102	0.755	0.141	0.274	0.465	3.6		13.03	1.5		
May 11	10.88	760	9.160	0.103	9.160	0.136	0.277	0.424	3.6		13.0	1.5		
											Fasting	1.5		

*The urine was acid throughout the day.

* The urine was acid throughout the experiment.

TABLE III.
Dog 31.

DATE 1913	WEIGHT	URINE*							N IN FOOD	PHLORHIZIN INJECTED
		Volume	Specific gravity	Total N	Creatinine N	Body N lost	Creatine N	Creatinine		
	kilos	cc.		grams	gram	grams	gram	gram	grams	grams
May 30	5.9	425	1006	2.092	0.060	2.092		0.161	Fasting	0
May 31	5.8	385	1006	1.845	0.054	1.845	0.008	0.145	Fasting	0
June 1	5.6	860	1008	3.224	0.052	3.224	0.032	0.140	Fasting	1.2
June 2	5.54	800	1011	4.170	0.052	4.170	0.080	0.140	Fasting	1.2
June 3	5.46	980	1008	4.279	0.053	4.279	0.103	0.143	Fasting	1.2
June 4	5.40	1420	1007	4.000	0.052	4.000	0.116	0.140	Fasting	1.2
June 5	5.10	1790	1004	4.452	0.050	4.452	0.123	0.135	Fasting	1.2
June 6	5.02	1540	1006	5.354	0.051	1.294	0.125	0.136	4.06	1.2
June 7	5.00	1740	1005	4.734	0.045	0.674	0.078	0.120	4.06	1.2
June 8	4.84	1580	1006	3.833	0.041	3.833	0.038	0.110	Fasting	1.2
June 9	4.72	1140	1005	3.255	0.035	3.255	0.025	0.094	Fasting	1.2

* The urine was acid throughout.

TABLE IV.
Dog 30.

DATE 1913	WEIGHT	URINE*								N IN FOOD	PHLORHIZIN INJECTED
		Volume	Specific gravity	Total N	Creatinine N	Body N lost	Creatine N	Creatinine	Creatine		
	kilos	cc.		grams	gram	grams	gram	gram	gram	grams	grams
May 15	8.70	136	1023	2.537	0.098	2.537	0.039	0.264	0.121	Fasting	0
May 16	8.60	70	1044	2.678	0.094	2.678	0.061	0.253	0.191	Fasting	0
May 17	8.40	350	1035	5.598	0.096	5.598	0.096	0.258	0.300	Fasting	1.2
May 18	8.20	360	1044	7.554	0.083	7.554	0.164	0.224	0.511	Fasting	1.2
May 19	7.90	440	1040	7.530	0.072	7.530	0.218	0.193	0.682	Fasting	1.2
May 20	7.72	580	1039	11.105	0.080	1.805	0.234	0.214	0.730	9.3	1.2
May 21	7.64	670	1032	11.105	0.078	1.805	0.199	0.211	0.622	9.3	1.2
May 22	7.40	680	1027	7.892	0.075	7.892	0.186	0.203	0.581	Fasting	1.2
May 23	7.10	600	1024	6.200	0.069	6.200	0.186	0.186	0.581	Fasting	1.2
May 24	6.96	640	1021	5.890	0.061	5.890	0.196	0.164	0.612	Fasting	1.2

* The urine was acid throughout.

totally independent of body tissue destroyed. The creatinine elimination shows the same general decrease from day to day as was noted in experiment I.

Tables III and IV detail two further experiments in this connection. In neither case were we able to get the animal to eat enough protein to completely overcome loss of some body nitrogen, but in both cases the nitrogen loss is brought down to well below the fasting level, with no demonstrable effect upon the output of creatine. Where the creatine drops noticeably on the first or second day of feeding it should be remembered that this often occurs whether the animal is fed or not, as illustrated by the continued drop if the food be withdrawn.

We believe that the experiments recorded in Tables I to IV demonstrate that the output of urinary creatine may be entirely independent of muscle disintegration, in the sense in which this latter term is usually employed. They leave open, however, the possibility that the creatine may have originated from preformed creatine in the muscular tissue by selective liberation. To study this point we have carried out the experiments detailed in Tables V and VI, where the dogs were completely phlorhizinized and were fed a liberal, but not excessive, amount of creatine-free protein for a period of eight days. The daily metabolism was determined, and at the end of the tenth day after beginning the phlorhizin injection, the animals were killed by bleeding from the femoral artery under ether anaesthesia and the creatine content of samples of muscle determined.

The experiments recorded in Tables V and VI serve to bring out strikingly the lack of relationship between body nitrogen lost and creatine output. They also serve to show that the drop in creatinine elimination recorded in the earlier experiments was primarily associated with loss of living tissue, and not with a depletion of initial body creatine, since, in those experiments where the loss in body weight was much less, both relatively and absolutely, the creatinine elimination drops very little. Conversely, we believe that the very slight drop in creatinine elimination shows that the body tissue was spared in the true sense of the word.

An analysis of the muscle tissue of dogs 36 and 39 showed, in both cases, a creatine content markedly in *excess* of the normal

Origin of Urinary Creatine

TABLE V.
Dog 36.

DATE 1913	WEIGHT		URINE*								N IN FOOD	PHLORHIZIN INJECTED
	Volume	Specific gravity	Total N	Creatinine N	Body N lost	Creatinine N	Creatine N	Creatinine	Creatine	D: N		
	cc.		grams	gram	grams	gram	gram	gram	gram		grams	grams
Nov. 17	13.96		3.473	0.153	3.473			0.410			Fasting	0
Nov. 18	13.70	1045	3.765	0.150	3.765			0.405			Fasting	0
Nov. 19	13.56	1039	9.396	0.155	9.396		0.038	0.418	0.117	4.2	Fasting	1.2
Nov. 20	13.28	1033	11.640	0.143	11.640		0.187	0.386	0.582	3.8	Fasting	1.2
Nov. 21	13.12	1042	20.130	0.137	2.590		0.232	0.371	0.713	3.5	17.54	1.2
Nov. 22	12.98	1040	19.450	0.136	1.910		0.130	0.368	0.402	3.4	17.54	1.2
Nov. 23	12.84	1032	20.065	0.131	2.525		0.151	0.352	0.472	3.4	17.54	1.2
Nov. 24	12.78	1025	20.290	0.149	2.750		0.158	0.400	0.495	3.2	17.54	1.2
Nov. 25	12.50	1022	20.070	0.143	2.530		0.167	0.386	0.520	3.3	17.54	1.2
Nov. 26	12.36	1026	19.780	0.127	2.240		0.156	0.341	0.493	3.1	17.54	1.2
Nov. 27	12.12	1033	21.640	0.124	4.100		0.170	0.334	0.529	2.9	17.54	1.2
Nov. 28	11.90	1024	24.935	0.125	1.615		0.200	0.338	0.618	3.2	23.32	1.2

* The urine was acid throughout.

TABLE VI.
Dog 39.

DATE 1914	WEIGHT	URINE*								N IN FOOD	PHOSPHORIN INJECTED
		Volume	Specific Gravity	Total N	Creatinine N	Body N lost	Creatinine N	Creatinine	D:N		
	kilos	cc.		grams	gram	grams	gram	gram		grams	grams
Jan. 6	8.20	150	1029	3.337	0.079	3.337	0.055	0.213		Fasting	0
Jan. 7	7.84	90	1014	3.345	0.077	3.345	0.090	0.208		Fasting	0
Jan. 8	7.62	70	1047	2.740	0.075	2.740	0.075	0.202		Fasting	0
Jan. 9	7.58	310	1043	6.339	0.074	6.339	0.110	0.200	3.9	Fasting	1.2
Jan. 10	7.44	500	1046	11.915	0.071	1.21	0.154	0.191	3.4	12.006	1.2
Jan. 11	7.30	620	1040	12.253	0.069	2.13	0.135	0.187	3.5	12.006	1.2
Jan. 12	7.16	650	1040	12.280	0.071	2.16	0.138	0.191	3.4	12.006	1.2
Jan. 13	7.08	700	1036	12.785	0.070	1.23	0.131	0.188	3.2	13.456	1.2
Jan. 14	7.02	960	1030	12.755	0.071	1.20	0.117	0.191	3.0	13.456	1.2
Jan. 15	7.04	930	1027	12.280	0.074	0.73	0.084	0.200	3.0	13.456	1.2
Jan. 16	7.10	860	1026	12.540	0.075	1.00	0.068	0.202	3.2	13.456	1.2
Jan. 17	6.94	800	1030	11.800	0.071	0.32	0.062	0.191	3.1	13.456	1.2
Jan. 18	6.94	710	1032	12.350	0.073	0.80	0.077	0.198	3.0	13.456	1.2

*The urine was acid throughout.

figure. Myers and Fine record the average creatine content of the fresh muscle of normal dogs at 0.37 per cent. The muscle from dog 36 showed a creatine content of 0.417 per cent, that from dog 39 showed a creatine content of 0.402 per cent, both calculated upon the fresh tissue. These figures serve to demonstrate beyond any argument that the creatine eliminated by these phlorhizinized animals did not represent creatine preformed in the muscle, and that muscle "disintegration" played no part in furnishing the creatine put out. We may explain the increased creatine present in the muscles of our animals upon the basis of a marked increase in the creatine circulating in the body fluids, because of a decreased power on the part of the body to destroy creatine. The fact that an administration of creatine to normal rabbits apparently causes slight increase in muscle creatine as shown by Myers and Fine is in harmony with this view.

TABLE VII.
*Analysis of muscle tissue.**

NUMBER OF DOG	PROTEIN	ETHER EXTRACT	MOISTURE	ASH	NITROGEN	CREATINE IN FRESH TISSUE	CREATINE IN ASH-, FAT- AND WATER-FREE TISSUE
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
36	20.15	1.6	78.2	1.05	3.22	0.417	2.11
39	18.94	3.08	77.2	0.92	3.03	0.402	2.13

* Fifty grams of muscle from the hind legs were used for each creatine determination, the tissue being extracted repeatedly with N 0.01 acetic acid. The filtrates were combined, concentrated, and the creatine determined after conversion to creatinine. All analyses were made in duplicate.

We have added in Table VII a detailed analysis of the muscle tissue of dogs 36 and 39. It may be of interest to note that in these animals the creatine content of the muscle upon an ash-, fat- and moisture-free basis very closely approximates the figures reported by Mendel and Rose for the creatine content of normal rabbit's muscle, calculated upon a similar basis. This point is of interest in connection with the data brought out by Myers and Fine in relation to the muscle creatine of certain species, and the creatinine coefficient of that species.¹³ Thus, in the rabbit, they found a muscle creatine content of 0.52 per cent and a creatinine

¹³ Myers and Fine: this *Journal*, xiv., p. 18 *et seq.*

coefficient of 14.3; while for man and the dog they reported a muscle creatine content of 0.39 per cent and 0.37 per cent, respectively, and a creatinine coefficient of 9 and 8.4, respectively.¹⁴

These parallelisms between creatine content of the muscle of a species and the creatinine coefficient for that species Myers and Fine interpreted as showing that the creatinine in the urine has as its precursor the creatine in the muscle. We agree with Myers and Fine that the analogy they cite in this connection is well worthy of consideration, and the line of reasoning suggested by it is very attractive. The conclusion they draw seems, however, hardly justifiable when we take into account how uncertain a factor is the so-called "creatinine coefficient." We know that in comparing different individuals of the same species the creatinine coefficient is subject to very wide variations, and we know that these variations are largely due to the different relative quantities of adipose and muscular tissue making up the body weight. To make a direct comparison of the creatinine coefficients in different species without due allowance for the influence of adipose tissue, as Myers and Fine have done, seems to us quite unjustifiable. From analyses it appears that the dog has from five to ten times as much fat in the muscle tissue as has the rabbit, and if any such relationship holds for total body weight, we should have to seek no further for an adequate explanation of the higher coefficient in the rabbit. To us it would seem that a better method of comparison between different species might be to determine the ratio of creatinine nitrogen to total nitrogen, *when the individual is at the lowest possible level of nitrogen excretion, i.e., is upon a practically nitrogen-free, carbohydrate diet.* Under such conditions the total nitrogen serves to indicate the total amount of *living* tissue in the body. Now if creatinine is an index of muscle creatine, as Myers and Fine suggested, instead of an index of special metabolism which does not imply creatine as a necessary precursor, one should expect the species in which there is most muscle creatine to show a higher ratio of creatinine to total nitrogen, when the total nitrogen is reduced to its lowest level, since neither the muscle creatine, nor the creatinine eliminated are appreciably affected.

¹⁴ The average from eleven dogs which we have had under observation for long periods, upon creatine- and creatinine-free diets, yields a creatinine coefficient for this animal of 10.2. We think that the figure 8.4 is decidedly too low as representing the creatinine coefficient for this species.

Using such a basis of calculation, and comparing man and the rabbit, we note that in man, upon a minimal nitrogen output, the creatinine nitrogen represents approximately 12-15 per cent of the total nitrogen.¹⁵ In the rabbit very few figures are available; but we may take as an example those reported by Mendel and Rose¹⁶ upon a rabbit which voluntarily ate loaf sugar until the creatine disappeared from the urine, and the total nitrogen fell to a minimum. In rabbit 6 we note that on February 16 the total nitrogen reached its lowest level. Comparing the ratio of creatinine nitrogen to total nitrogen in this and the following five days, we note that the creatinine nitrogen represents 12.1 per cent of the total nitrogen, a figure somewhat *lower* than the average figures reported for man by Folin.

This basis of calculation seems to speak against the view that creatinine is an index of muscle creatine. While the method of calculation may be open to some objections, it would seem to be theoretically more correct than the one employed by Myers and Fine in this connection.

The experiments detailed in the present paper are probably the only ones so far presented which definitely show that creatine may be eliminated in the urine in large amounts without any corresponding loss of body tissue or of preformed creatine from the muscular tissue. In this connection the question arises as to whether the conclusions drawn would hold for the creatine eliminated during simple starvation. While we believe that such is the case, we also feel that the experimental evidence so far available is not conclusive.¹⁷ It is interesting, however, to note that in simple starvation the quantity of creatine eliminated is small, and that this creatine completely disappears upon feeding pure carbohydrate. Our experiments have dealt primarily with *complete carbohydrate starvation*, which is, in one respect, a greatly intensified simple starvation; and under such conditions the creatine output is not influenced by the ingestion of food, so long as carbohydrate utilization is prevented. It will be of interest to discuss at this point the experimental evidence which has so far

¹⁵ Cf. Folin: *Amer. Journ. of Physiol.*, xiii, p. 66, 1905.

¹⁶ Mendel and Rose: *this Journal*, x, p. 226, 1911.

¹⁷ Further evidence in this connection will be presented in a subsequent communication.

been offered in support of F. G. Benedict's original hypothesis that the creatine of starvation is derived from the muscle tissue. As noted above, the recent work of Myers and Fine upon the creatine eliminated during starvation is the most complete which is available upon this point.

The assumption at the basis of the work of Myers and Fine, and of the conclusion which they draw is as follows. "It is reasonable to believe that if the creatine eliminated owes its origin to the disintegration of muscle tissue, the total creatine of the body will be depleted in proportion to the amount eliminated in the urine, provided creatine is not readily destroyed in the body." One of the chief difficulties we find in accepting the conclusions which Myers and Fine draw lies in the last portion of this assumption, viz., "provided creatine is not readily destroyed in the body." Myers and Fine have offered no experimental evidence to show that creatine is not readily destroyed in the body during simple starvation. The appearance of small quantities of creatine in the urine does not in the least demonstrate that much larger amounts may not be destroyed. A gram of sugar per day in the urine is not a complete diabetes, and we believe that direct experimental evidence is needed to justify an assumption that creatine is not destroyed to a very considerable extent in the fasting organism.

We believe that the attempt of Myers and Fine to account for "initial body creatine" is without special significance, since the origin, rôle and fate of creatine in the normal or fasting organism are still unknown factors.

Aside from the question of the initial body creatine, the work of Myers and Fine presents interesting data on the creatine content of muscle during starvation. Observations in this connection had been previously reported by Dorner,¹⁸ by Mendel and Rose¹⁹ and by Hawk and collaborators,²⁰ but Myers and Fine have given by far the most satisfactory data on this point. We question, however, whether the concentration of creatine in the muscle of the normal and of the starving animal can yield definite evidence as to the origin of urinary creatine. The data in this connection may be suggestive, but when we take into account the possible

¹⁸ Dorner: *loc. cit.*

¹⁹ Mendel and Rose: *loc. cit.*

²⁰ Hawk and Howe: *loc. cit.*

changes in the disintegrating muscle of its power to fix creatine, providing this substance is present in the circulating fluids, it is obvious that because the creatine present in the muscle has decreased during a fast we cannot conclude that the only or indeed the chief source of the urinary creatine was disintegrating muscle tissue. As a matter of fact, Myers and Fine report that in short fasts (up to ten days) the creatine content of rabbit's muscle increases by over 10 per cent, while for longer fasts (fifteen to thirty days) the muscle loses markedly in its content of creatine. To the writers such results appear flatly contradictory so far as affording explanation of the origin of urinary creatine is concerned, since creatine is eliminated throughout the whole period of starvation. Myers and Fine conclude that the decrease in the creatine content of the muscle tissue of animals subjected to a long fast shows that the urinary creatine has its origin in the muscle tissue, especially as animals which fast a long time excrete relatively and absolutely more creatine in the urine than do animals which fast only a short period. They explain the increase in the creatine content of the muscle of animals subjected to a short fast by the assumption of the removal of the non-creatine-containing portion of the muscle, *i.e.* glycogen, fat, etc., more rapidly than of the creatine-containing portion. This explanation lacks sufficient data to support it. The 11 per cent increase in creatine noted cannot be explained upon a simple assumption of removal of fat and glycogen. Reported analyses of rabbit's muscle show that there is not enough of these substances present to account for 50 per cent of the increase of creatine noted, even if they were completely removed.

We can apparently only conclude that the creatine content of rabbit's muscle is increased for short fasts, and decreased for longer fasts. A way of connecting this with the elimination of creatine in the urine during the same periods is not clear to us.

In the original hypothesis of Myers and Fine we note the phrase that the urinary creatine owes its origin to the "disintegration" of muscle tissue. It is not clear in just what sense this term disintegration, is employed. Obviously the creatine might come from muscular tissue completely catabolized, the creatine being set free, and appearing in the urine; or it might be due to this process, as well as to a selective loss of creatine from muscular tissue remaining

otherwise essentially intact. If the first of these suppositions be correct, the total nitrogen of the urine during starvation should show an amount of muscular tissue destroyed closely approximating the creatine (or perhaps the creatine plus creatinine) in the urine. Mendel and Rose called attention to the fact that the total creatinine in the urine closely parallels the total nitrogen. Myers and Fine have also employed this calculation, and have shown that in many instances a calculation of muscle destroyed on a basis of total creatinine eliminated yields a figure very close to that obtained from the total nitrogen put out in the urine. Such calculations are suggestive, but cannot be regarded as conclusive, especially as figures are available showing a wide variation when such a basis of calculation is employed.²¹ It may be added that the method of calculation employed by Mendel and Rose and by Myers and Fine must be based upon the view that the creatinine and creatine contained in the urine represent completely catabolized muscular tissue, and it is hard to understand why Myers and Fine should use this method of calculation to prove the common origin of creatine and creatinine in muscular tissue, and then expect to find the percentage of creatine in the remaining muscular tissue decreased. If the creatine which is lost from the otherwise intact muscle appears in the urine, together with that set free from completely catabolized muscular tissue, the calculation used by Myers and Fine should show considerably more muscular tissue destroyed on a basis of the creatine-creatinine content of the urine, than on a basis of the total nitrogen put out. As a matter of fact their figures show in some cases a very close agreement, while in many instances those on a basis of the creatine-creatinine calculation are appreciably *lower* than where the total nitrogen factor is employed.

SUMMARY AND CONCLUSIONS

Experiments have been reported showing that in the dog a high creatine elimination in the urine may be maintained which is wholly independent of body tissue destroyed. Furthermore, it has been shown by analyses of the muscle tissue of such animals, that the creatine eliminated did not have its origin in preformed creatine of the muscular tissue.

²¹ Wolf and Osterberg: *Amer. Journ. of Physiol.*, xxviii, p. 71, 1911.

Creatine is probably being formed in the animal organism in relatively large amounts, and is normally for the most part either utilized or destroyed.²² If creatinine is an end product of creatine metabolism, the quantity of the former substance eliminated can account for only a small proportion of the creatine normally metabolized. Whether creatine is, for the most part, normally utilized or destroyed we have no means of telling at present, but it seems safe to conclude that the power of the organism to metabolize the creatine which it forms is directly related to certain processes, chief among which appears to be the utilization of carbohydrate, as suggested by Cathcart and by Mendel and Rose.

Some of the recent work of Myers and Fine upon creatine and creatinine metabolism has been discussed.

In conclusion, we may state that we are continuing the work upon creatine formation in phlorhizinized animals, as well as the fate of creatine and creatinine when parenterally introduced into such animals. It would appear that the phlorhizinized animal should offer a new and valuable field in which to study creatine formation. Under ordinary circumstances creatine formed in the body is probably largely or completely retained or destroyed, so that to administer a possible creatine precursor and look for creatine in the urine is probably about as unprofitable as to administer lactic acid to a normal animal and look for dextrose in the urine. The phlorhizinized animal is seemingly for a time at least, practically "diabetic" as regards creatine destruction, and we hope that studies based upon such observations will yield valuable data.

²² In this connection see also the recent paper of Kraus: *Quart. Journ. of Exp. Physiol.*, viii, p. 87, 1913.

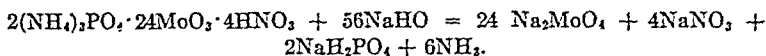
ON THE ESTIMATION OF PHOSPHORUS IN BIOLOGICAL MATERIAL.

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The well known Neumann method for the titrimetric estimation of phosphoric acid is based upon the following equation:



Essential to the successful precipitation of the yellow compound in the desirable coarse form are high temperature of the solution, a slight excess of ammonium molybdate, a large excess of ammonium nitrate and a distinctly acid reaction. Under the most favorable circumstances precipitation is almost entirely complete and is accomplished within a few minutes to a half hour. The presence of more than nominal amounts of certain neutral salts leads to a distinct increase in solubility of the precipitate; the chlorides in particular act thus, excess of sulphuric acid and sulphates also to some extent. As the method was elaborated by Neumann, using large amounts of material for analysis, these factors were negligible; but when one wishes to estimate minute amounts of phosphoric acid, the disturbing factors must be eliminated. We have been able to show, as have all other observers before us, that the precipitate formed when phosphoric acid, or phosphates of the common metals in aqueous solution, is heated in the presence of an excess of ammonium nitrate and ammonium molybdate corresponds to the above stated equation in so far as the amounts of phosphoric acid, molybdenum, alkali and ammonia are concerned. The nitrate is in practice in part substituted by sulphate.

When the equation is contemplated, it is evident that one might base a quantitative determination upon the weight of the

whole molecule of precipitate, upon a titration with alkali (as is done in the Neumann method), upon an estimation of the ammonia and finally upon the estimation of the molybdenum. Essential in all would be the complete washing out of the excess of ammonium nitrate, ammonium molybdate and acid of the solution. Tempting though it be when one regards the marked disproportion between the weight of the total molecule of precipitate and the weight of PO_4 contained therein, a gravimetric estimation is not practicable—firstly, because of the fluctuation between nitrate and sulphate in the precipitate, and secondly, because the precipitate cannot be dried without disintegration. Estimation by determining the ammonia is feasible, but not so rapid as by titration, if the amount involved be large enough to permit of an accurate titration. Estimation of the molybdenum, colorimetrically, is finally feasible especially for minute quantities, and will be later described.

Neumann in his publication¹ states that 0.015 gram of P_2O_5 may be accurately determined by titration as described by him. As a matter of fact, with the aid of skilful manipulation in the washing, 0.001 gram of P_2O_5 may be accurately estimated by titration. Under these circumstances, the estimation of the total phosphorus of the urine is done as follows:

The reagents are a clear 10 per cent solution of pure ammonium molybdate in 25 per cent sulphuric acid; powdered sodium carbonate or a saturated solution of same; powdered ammonium nitrate; nitric acid. Two cc. of urine are measured with an Ostwald pipette into a platinum dish, 2 drops of saturated solution of sodium carbonate added and the mixture carefully evaporated to dryness. When dry, the mass is ashed in the direct flame, a red heat being sufficient for complete ashing. This is more rapid than the wet method of ashing according to Neumann, and in particular avoids the excess of salts and acid in the solution to be precipitated. The fused mass in the dish is then taken up with about 5 cc. of dilute nitric acid and the solution quantitatively transferred to a 50 cc. centrifuge tube. The platinum dish is thrice washed with not to exceed 3 cc. of distilled water. The total bulk in the centrifuge tube should not be over 15 cc.

¹ Neumann: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 133.

Solid ammonium nitrate is now added to make the concentration 15-20 per cent and the tube placed in a water bath. When the bath is boiling, 5 cc. of the ammonium molybdate solution are run directly into the contents of the tube and gently mixed. The solution of ammonium molybdate should not be run in along the side of the glass tube, but directly into the center of the solution, since otherwise it may later precipitate and cling to the walls of the tube. After standing in the boiling water bath for from ten to thirty minutes, the tube is taken out and manipulated in such a manner as to drive the precipitate from the sides and surface of the solution. If the yellow precipitate were to be centrifuged directly it would be found that a small portion would remain upon the surface, due to the factor of surface tension. The precipitate must be washed down with something that lowers this surface tension and at the same time is not a solvent of the precipitate. Fifty per cent alcohol meets these requirements. The alcohol should be prepared from pure absolute alcohol since commercial grades of strong alcohol are more or less acid in reaction. For the first two washings absolute alcohol must be used. Ten cc. of absolute alcohol are carefully poured down the side of the tube, care being taken to wash the entire sides. The alcohol carries the clinging yellow precipitate into the fluid; if now this be gently mixed, it will be found possible to fill the tube with 50 per cent alcohol as a superimposed layer. When centrifugated, the entire yellow precipitate will be packed in the bottom of the tube and the supernatant fluid can be poured off cleanly. Inspection of the sides of the tube will indicate however that some of the yellow precipitate clings. To free this portion, we proceed as follows. About 5 cc. of distilled water are poured in and the sides carefully rubbed clean with a rubber-capped rod, which is then washed clean into the tube with an equal volume of absolute alcohol. This washing with water has also the effect of dissolving any molybdate or nitrate that may have separated out, or been held down beneath the coating of yellow precipitate along the wall. Both ammonium nitrate and molybdate are freely soluble in neutral 50 per cent alcohol. The sides of the tube are then again carefully washed down with the dilute alcohol and when this is accomplished, the tube is again filled to about three-fourths by floating with dilute alcohol as

previously described. The tube is again centrifugated, and the yellow precipitate will be found packed, the sides clean and the surface free of precipitate. The supernatant fluid is poured off the precipitate, about 10 cc. of dilute alcohol poured in, the precipitate thoroughly shaken by swinging the tube, and again filled up as before with dilute alcohol. This washing by centrifugal decantation must be repeated until the reagents are removed and the wash alcohol is neutral. Five washings after the first are sufficient. A certain knack is required to effect the washing down with the alcohol in such a manner as to enable the centrifugal action to carry down all the yellow precipitate, leaving none on the surface of the fluid or upon the surface of the glass above the fluid. A little practice will enable one to so conduct this operation as to pack all the precipitate in the bottom of the tube, so that the clear wash alcohol may be poured off. One should always note the appearance of the yellow precipitate. If it be tinged with white, this indicates precipitation of oxide of molybdenum and the test must be rejected. The yellow precipitate is practically insoluble in 50 per cent alcohol in the time occupied in the washing as we have proved by repeated analysis of such alcoholic washings.

Five cubic centimeters of the molybdate solution is enough for 5 mgm. of P_2O_5 in the 2 cc. of urine. If the urine be greatly concentrated or the amount of phosphoric acid high, less urine should be used rather than more molybdate. Care must be taken not to exceed the capacity of the centrifuge tube, since over half of the capacity of the tube must be reserved for the washing fluid.

The tube containing the washed precipitate is now placed under the burette of decinormal or twentieth-normal alkali, and enough added to bring the precipitate entirely into colorless solution, and the amount added noted. We usually run in 10 to 20 cc. or any round number in excess of the amount needed for solution. The ammonia must now be driven off. This cannot be done by boiling the centrifuge tube, since spattering is almost certain to lead to loss. The contents may be quantitatively transferred to Erlenmeyer flasks and the ammonia boiled off, but unless carefully watched even in these spattering is certain to occur from the alkaline solution. The best method is to evaporate the contents to dryness at a temperature below the boiling point of water.

We do this in a dry sand bath which may be incidentally described here, since to judge from comments passed upon it at the recent meeting of the Society of Biological Chemists in this city, the bath seems to be new.

The bath consists of steam radiators placed horizontally in a hood, and covered with sand. Depending on whether the radiator is fed with high or low pressure steam, the temperature will be more or less elevated. With ten pounds of steam, the sand just above the coils will have a temperature of over 100°C . decreasing as the layer of sand is thickened above the radiator. Since the atmosphere within the hood is dry, evaporation proceeds much more rapidly than from a water bath and the use of the bath over night carries no danger.

The tubes or flasks are placed in this sand bath over night. On the following morning the contents are dry. The white coating is then dissolved in a little hot water and titrated to neutrality with any sensitive indicator, the difference between the amount of alkali added and acid required to reach neutrality corresponding to phosphoric acid, in accordance with the equation above given. One cubic centimeter of decinormal solution corresponds to 0.0002534 gram (in theory) which figure should be rounded to 0.00025 gram since the last two figures correspond to the error.

The method when properly carried out gives good checks, with duplicates, on urine, feces, and with phosphate solutions. But with known phosphate solutions, through the duplicates check, the results are always a little too high. This is doubtless due to occlusion of ammonium molybdate or ammonium nitrate in the yellow precipitate, depending partly upon the density of the precipitate. The results run from 1 to 2 per cent too high. Under these circumstances, the rounding off of the figure of calculation from 0.0002535 to 0.00025 gram tends to balance the error. Too high results are also obtained when the final estimation is done by measurement of the ammonia evolved in the reaction of neutralization.

To estimate the so-called neutral phosphorus of the urine, 20 cc. of urine are made alkaline by the addition of powdered barium hydrate, filled to 25 cc. and filtered, the precipitate containing barium phosphate and barium sulphate. 20 cc. of the filtrate are then made acid by addition of sulphuric acid, filled to 25 cc. and again filtered. 20 cc. of this filtrate are then evaporated to

dryness and ashed, as before described. In this ash, the phosphoric acid derived from the neutral phosphorus of the urine is then estimated in the same manner as described, and the result calculated in accordance with the portions used in the several steps. More urine may be employed if necessary, should the neutral phosphorus, as sometimes, be very low. The difference between the figure for total phosphorus and neutral phosphorus represents the ionized phosphate of the urine or feces. It is not permissible to estimate the ionized phosphate by any other method, and by subtraction from the total phosphorus arrive at a figure for the neutral phosphorus, since the error falls entirely on the neutral phosphorus and would greatly modify the figure for this small fraction.

Feces we ash dry, in the same manner. A gram of dried feces is mixed with about 0.1 gram of dried sodium carbonate, and the mass ashed carefully, under the direct flame. The fusion mass is then dissolved in dilute nitric acid and made up to a known volume. With a small amount of this known solution, as orientation test is done to determine the approximate amount of phosphoric acid present. The practiced eye can gauge a milligram of P_2O_5 in the yellow precipitate quite closely. The known amount is then carried through the method as described. It is not possible in feces to separate the neutral from the ionized phosphoric acid, as described in the urine.

The method is quite rapid, for despite the many manipulations in the washings, these proceed rapidly, if properly performed, since the heavy precipitate centrifugates very rapidly. Using a centrifuge with a capacity of eight 50-cc. tubes, four estimations in duplicate are easily carried out. As a routine procedure it is not necessary to carry out the estimations in duplicate.

Minute traces of phosphoric acid may be estimated in an approximate manner by means of the colorimeter. The molybdenum in the complex precipitate with phosphoric acid reacts with certain reducing agents to yield lower oxide stages of the metal of a blue color. There is quite a literature on the oxide stages of molybdenum and the conditions of the reactions of reduction through which they are attained. It would not be profitable to enter upon this discussion here, since the method must rest upon an empirical foundation, because there is no known relation of the

different oxide stages to the degrees of blue color they exhibit. It has been necessary to work out the conditions empirically. Colorimetric estimations as a rule rest upon the reaction $A + B$, with the production of a color associated with the reaction in a quantitative sense, either A or B being the unknown, the other being present in the reaction in excess. In the Folin colorimetric estimation of uric acid, the metal to be reduced is present in excess, and the reduction is used to measure the amount of the reducing material (uric acid) present. In the present instance, on the other hand, the metal to be reduced is the unknown quantity to be measured, the reducing agent is present in excess. It would not be profitable to describe the numerous qualitative and quantitative studies that were carried out in examining the conditions of reduction as applied to the task in hand; the results alone will be given. It has been clearly established that to carry on the reduction of the molybdenum in the phosphate precipitate in such a manner as to enable one to compare the color with that of a known reduction of the same metal, it is necessary that the reaction occur in the cold and in acid solution. The best reducing agent we have found to be phenylhydrazine, originally suggested by MacCallum.² Hydrazine sulphate and chloride reduce, but in a less controlled manner. The solution of phenylhydrazine hydrochloride must be fresh, or at least not over one week old. We use a 5 per cent solution, which should be colorless.

The control solution must contain both phosphoric acid and molybdenum oxide in proper proportions for combination. We have tried to use simple solutions of molybdic acid or salts, but with results far inferior to those obtained when the phosphate-molybdenum precipitate is reduced. Relatively pure molybdic acid may be obtained on the market, containing about 85 per cent of MoO_3 ; and ammonium molybdate containing about 80 per cent MoO_3 . These must be assayed for their actual value of MoO_3 . A $\frac{\text{Mol}}{150}$ solution of disodium hydrogen phosphate contains in the cubic centimeter 0.000206 gram P. This is the standard solution of phosphate we employ. (Incidentally remarked, this solution must, in this climate, be made up with the use of a salt

² MacCallum: *Proc. Roy. Soc.*, lxiii, p. 467.

containing *no* water of crystallization.) In the yellow precipitate of phosphate with molybdenum oxide are 12 atoms of Mo to one atom of P. Therefore the molybdenum solution should be made up so that in a small unit volume (1 cc.) is contained the amount of MoO_3 necessary to unite with 0.0002 gram P. This will require from 11 to 12 grams of MoO_3 and from 13 to 14 grams of ammonium molybdate to the liter, depending upon the preparation in hand, analysis being the basis of exact calculation for the preparation of the standard solution.

The use of a method for the estimation of minute amounts of phosphoric acid does not lie in application to substances that, like urine, contain it in large amounts. It is only of use with material that contains but small amounts of P and available in but small quantities for analysis, as blood serum, cerebro-spinal fluid, etc. The material must first be ashed. When choice is permissible, we much prefer dry ashing in the presence of sodium carbonate. Sometimes this is not possible, then wet ashing must be used. We have often used for analyses the final residue of the test for non-protein nitrogen in the blood serum, according to Folin; after the ammonia is removed the residual fluid is submitted for precipitation of its contained phosphoric acid. The phosphoric acid is precipitated as above described. Now, dealing with minute amounts and especially in the presence of an excess of salts, precipitation is often long delayed, and tends to be incomplete. We secure complete precipitation of the phosphoric acid either by allowing the tube to stand in a hot bath over night, or by evaporating the contents of the tube to dryness. If after standing over night, the yellow precipitate at the bottom is covered by a clear and colorless supernatant fluid, precipitation may be regarded as approximately complete. If the fluid be yellow, this is not the case, and the contents of the tube must be evaporated to dryness. Then if the residue be taken up in a small amount of warm water acidulated with nitric acid, the yellow precipitate that would not before crystallize out of solution will usually not pass back into solution. Rarely this is not the case, and under these circumstances an estimation cannot be done. As minute an amount of P (in disodium hydrogen phosphate) as 0.000005 gram will give a distinct yellow color, and this is the test for complete precipitation. The precipitate is washed as previously described. If the amount of salts be not large, it should

be first washed as previously described. If the amount of salts be very large, it should be first washed with a dilute alcohol acidulated with nitric acid until the offending excess of chlorides or sulphate is removed, thereafter the washings are continued with the neutral dilute alcohol.

After the precipitate is washed, it is dissolved in 5 cc. of tenth-normal sodium hydroxide, and the solution transferred quantitatively to a 25 cc. flask. Into a second such flask are placed 1 cc. of the solution of disodium hydrogen phosphate containing 0.00026 gram P (with Ostwald pipette), the fixed amount of the molybdenum solution containing the exactly corresponding amount of MoO_3 and 5 cc. of decinormal sodium hydroxide. To each flask are then added 2 cc. of the solution of phenylhydrazine, and then to each flask 10 cc. of decinormal sulphuric acid, and the flasks filled to the mark with distilled water. The blue colors slowly develop and are complete in a half hour, when the colors are compared in a Duboscq colorimeter, the standard being of course the basis of the reading. If the amount of phosphorus be very small, the standard color solution may be diluted to a fifth, and the reading made with that as a standard. The colors do not fade rapidly, but do tend to assume a purplish tinge. Usually the two colors are of the same kind of blue. Occasionally the unknown does not present the normal color, and then the estimation is to be rejected.

It is possible in this way to estimate in an approximate manner very small amounts of phosphoric acid. The following figures afford an illustration of the results.

Used:	Recovered:
0.00042.....	0.00050
0.00103.....	0.00108
0.00082.....	0.00092
0.00048.....	0.00053

The values are always too high, a result due we believe to occlusion of reagents in the precipitate.

Duplicate estimations agree fairly well, as may be seen in the following findings, duplicates for different analyses.

0.00023 and 0.00020
 0.00025 and 0.00020
 0.00045 and 0.00041
 0.00046 and 0.00041

With this method we have found that blood serum contains, in the form of phosphorus in lipoidal combination, from 0.004 to 0.006 gram P to 100 cc. of serum. The serum does not contain enough phosphate, as salt, to give a clear qualitative test, this confirming the investigation of Gürber.³ In one instance we found the large figure of 0.014 gram P for 100 cc. of blood. It is possible that the lipoidal phosphorus of the blood is subject to notable pathological variations. We have not studied enough samples of cerebro-spinal fluid to enable us to make at the present writing definite statements on its lipoidal phosphorus content.

Since ammonia is present in the yellow precipitate in the proportion of 6NH_3 to 2P , it is obvious that the amounts of NH_3 present in the precipitates used colorimetrically for the estimation of the molybdenum content lie within the range of the colorimetric estimation of ammonia with Nessler's reagent according to Folin. It may be measured in this manner, but the procedure is less rapid than as described, since the driving-over of the ammonia is added to the series of necessary procedures. On the other hand, the preparation of the control reagents for the Folin-Nessler colorimetric test is more simple than is the preparation of the standard solutions of phosphates and of molybdenum oxide. We have made but few such estimations of ammonia, and are not prepared to express an opinion as to the comparative accuracies of the two methods of final estimation.

³ Gürber: *Verhandl. d. phys. chem. Gessellsch. Würzburg*, N. F., xxviii, p. 231.

STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

II. THE SYNTHESIS AND RATE OF ELIMINATION OF HIPPURIC ACID AFTER BENZOATE INGESTION IN MAN.¹

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(Received for publication, May 28, 1914.)

The question of the extent of the ability of the human organism to detoxicate benzoic acid by conjugation with glycocholic acid and elimination as hippuric acid in the urine has frequently been raised. Lewinski² observed in the urine of men to whom 12 to 20 grams of benzoic acid had been given in small doses during the course of twelve hours, neither free benzoic acid nor benzoyl glucuronates. With larger doses of from 25 to 40 grams, small amounts (up to 26 per cent of the intake) of free benzoic acid were present in the urine, which was also dextro-rotatory and contained reducing substances, *i.e.*, glucuronates. Thus with large doses, up to 20 grams of benzoic acid, the conversion to hippuric acid was found to be practically quantitative. Brugsch³ and Tsuchiya⁴ were unable to confirm the work of Lewinski and recovered only 22 to 48 per cent of the ingested benzoic acid (4 to 24.6 grams) as hippuric acid. Dakin⁵ found that benzoic acid taken by men in doses of 5 to 10 grams per day underwent a practically complete conversion to hippuric acid before elimination in the urine. No free benzoic acid could be detected in the urine. In none of these experiments was any difference between the free acid and the sodium salt observed.

¹ Presented in abstract before the Society for Experimental Biology and Medicine, June 6, 1914.

² Lewinski: *Arch. f. exp. Path. u. Pharm.*, lviii, p. 397, 1908.

³ Brugsch: *Zeitschr. f. exp. Path. u. Ther.*, v, pp. 731-6, 1909.

⁴ Tsuchiya: *Ibid.*, v, pp. 737-42, 1909.

⁵ Dakin: *this Journal*, vii, pp. 1913-8, 1909.

In a previous paper⁶ it has been shown, in confirmation of the work of McCollum and Hoagland⁷ on pigs, that in rabbits after the ingestion of sodium benzoate a decrease in the nitrogen eliminated as urea could be observed, a decrease closely corresponding to the amount of nitrogen eliminated as hippuric acid. From these experiments it appeared that the glycocoll available for conjugation was derived from some substance, whose nitrogen normally was eliminated as urea nitrogen, *i.e.*, a deviation from the path of normal metabolism.

Studies⁸ of the metabolism of man after benzoate ingestion had previously shown no changes in nitrogenous metabolism. It seemed however that because of the large amounts of total nitrogen eliminated in man in twenty-four hours, any slight changes in the elimination of urea, due to a deviation of small amounts of nitrogen from the normal path of catabolism to the synthesis of hippuric acid, might be obscured. With this in mind a study of the relations between the elimination of urea and of hippuric acid during two-hour periods has been made in the belief that if the human organism resembled that of the pig or rabbit, in regard to the synthesis of hippuric acid, the excretion of hippuric acid nitrogen at some period of time might be sufficiently great to result in a decreased urea nitrogen elimination.

The subject, a healthy man, 86 kilos in weight, was maintained on a diet of milk (1050 cc.), cane sugar (180 grams), and butter (75 grams) daily for three days. This diet was low in protein content and contained no glycocoll. The urine was collected at two-hour intervals throughout the day with night periods of twelve hours. Nitrogen was determined by the Kjeldahl-Gunning method, urea by the method of Benedict, hippuric acid by the method of Folin and Flanders, and free benzoic acid by the method of Dakin. At 8.30 on the morning of the second day 10 grams of sodium benzoate (equivalent to 8.47 grams of benzoic acid) were taken dissolved in water with the food.

The urea + ammonia nitrogen of the urine during the first three two-hour periods (VIII-X) following the ingestion of the benzoate shows a marked decrease in comparison with the corre-

⁶ This *Journal*, xvii, pp. 193-8, 1914.

⁷ McCollum and Hoagland: this *Journal*, xvi, p. 321, 1913.

⁸ U. S. Dept. of Agriculture, Report No. 88, p. 56, 1909.

sponding periods (I-III) of the previous day. In spite of the slightly higher elimination of total nitrogen in the experimental periods as compared with the normal periods, the urea + ammonia nitrogen is 0.088, 0.284, and 0.265 gram lower in the benzoate periods, than in the normal periods corresponding. As shown in the table, the maximum elimination of the hippuric acid takes place during these first six hours. After the elimination of the greater part of the ingested benzoate (86.8 per cent in the first

TABLE I.
Subject L.

PERIOD	ENDING	TOTAL N	UREA + AMMONIA N			UNDETERMINED N	TOTAL BENZOIC ACID		HIPPURIC ACID CAL- CULATED FROM BENZOIC ACID
								Percent of intake	
		grams	grams	per cent	gram	grams			grams
I	10.30 a.m.	1.134	0.974	85.9	0.160				
II	12.30 p.m.	1.242	1.086	87.4	0.156				
III	2.30 p.m.	1.145	1.014	88.6	0.131				
IV	4.30 p.m.	1.044	0.930	89.1	0.114				
V	6.30 p.m.	1.054	0.921	87.6	0.133				
VI	8.30 p.m.	0.986	0.848	86.0	0.138				
VII	8.30 a.m.	4.142	3.317	80.0	0.725	0.188			0.276
VIII	10.30 a.m.	1.304	0.886	67.9	0.418	2.029	23.9		2.979*
IX	12.30 p.m.	1.302	0.802	61.6	0.500	3.045	36.0		4.470
X	2.30 p.m.	1.154	0.749	64.9	0.405	2.280	26.9		3.347
XI	4.30 p.m.	No urine							
XII	6.30 p.m.	1.861	1.582	85.0	0.279	0.460	5.4		0.672
XIII	8.30 p.m.	1.381	1.251	90.6	0.130	0.079			0.116
XIV	8.30 a.m.	4.819	4.186	86.9	0.633	0.244			0.359

*At 8.30 a.m. 10 grams Na benzoate *per os*.

six hours), the urea + ammonia nitrogen elimination becomes normal again. A comparison of the elimination of the first six hours of the normal day with the corresponding period of the benzoate day illustrates the point clearly.

If from the undetermined nitrogen of the benzoate periods, the nitrogen eliminated as hippuric acid be subtracted, the resulting figure (shown in table II in parentheses) is comparable to the undetermined nitrogen of the normal period. From this experi-

ment it appears that in the human organism, as in that of the pig or rabbit, hippuric acid nitrogen may be derived at the expense of urea + ammonia nitrogen.

TABLE II.

PERIODS	TOTAL N	UREA + AMMONIA N		UNDETERMINED N	HIPPURIC ACID N
	grams	grams	per cent	grams	grams
I-III Normal.....	3.521	3.074	87.3	0.447	
VIII-X Benzoate.....	3.760	2.437	64.8	1.323 (0.481)	0.843

In the above experiment, although 10 grams of sodium benzoate were administered in a single dose, no free benzoic acid nor glycuronates could be detected in the urine. The urine did not reduce Fehling's solution either on heating, or in the cold after standing some hours. No toxic effects of the benzoate were noted. The organism was able to synthesize and eliminate nearly quantitatively within six hours the hippuric acid arising from 10 grams of sodium benzoate. A study of the rate of elimination shows that 23.9 per cent of the ingested benzoate had been converted to hippuric acid and eliminated within two hours, 59.9 per cent at the end of four hours, and 86.8 per cent at the end of six hours.

In order to ascertain whether the liquid nature of the diet in the above experiment was a factor in the rapid absorption of the benzoate, synthesis into hippuric acid, and elimination in the urine, the experiment was repeated on the same subject on other diets.

As shown in table III, the synthesis and elimination of the hippuric acid on a mixed diet was even more rapid than on a milk, etc., diet. The percentages of the ingested benzoate eliminated in two, four and six hours were 37.4, 81 and 92.3 respectively. That is, four-fifths of the hippuric acid was eliminated within four hours. In the third experiment, during which the subject was on a purine-free diet, the percentage eliminations at the end of periods slightly more than one, three and five hours in length were 21.5, 62.4 and 87.2 respectively. All three of these experiments show an elimination as hippuric acid of 85-90 per cent of the ingested benzoate within from five to six hours. A similar experi-

ment was performed on another subject, on a mixed diet, to whom 6 grams of the benzoate were given. No free benzoic acid nor glycuronates could be detected in the urine. With this smaller dose the elimination had reached its maximum in three and one-half hours, at which time over 85 per cent of the hippuric acid had been eliminated.

TABLE III.

Subject L.

HOURS AFTER ADMINISTRATION	TOTAL BENZOIC ACID		HIPPURIC ACID CALCU- LATED FROM BENZOIC ACID	NOTES
		Per cent of intake		
	<i>grams</i>			
2	3.178	37.4	4.665	Mixed diet, no fruit. 10 gms. sodium benzoate <i>per os</i> 1 hr. after breakfast.
4	3.682	43.6	5.405	
6	0.959	11.3	1.401	
8	0.201		0.295	
10	0.102		0.150	
12	0.098		0.144	
24	0.755		1.108	

Subject L.

1 hr. 10 min.	1.821	21.5	2.673	Purine free diet, otherwise as above.
3 hr. 10 min.	3.465	40.9	5.087	
5 hr. 10 min.	2.105	24.8	3.090	
7 hr. 10 min.	0.061	0.8	0.090	

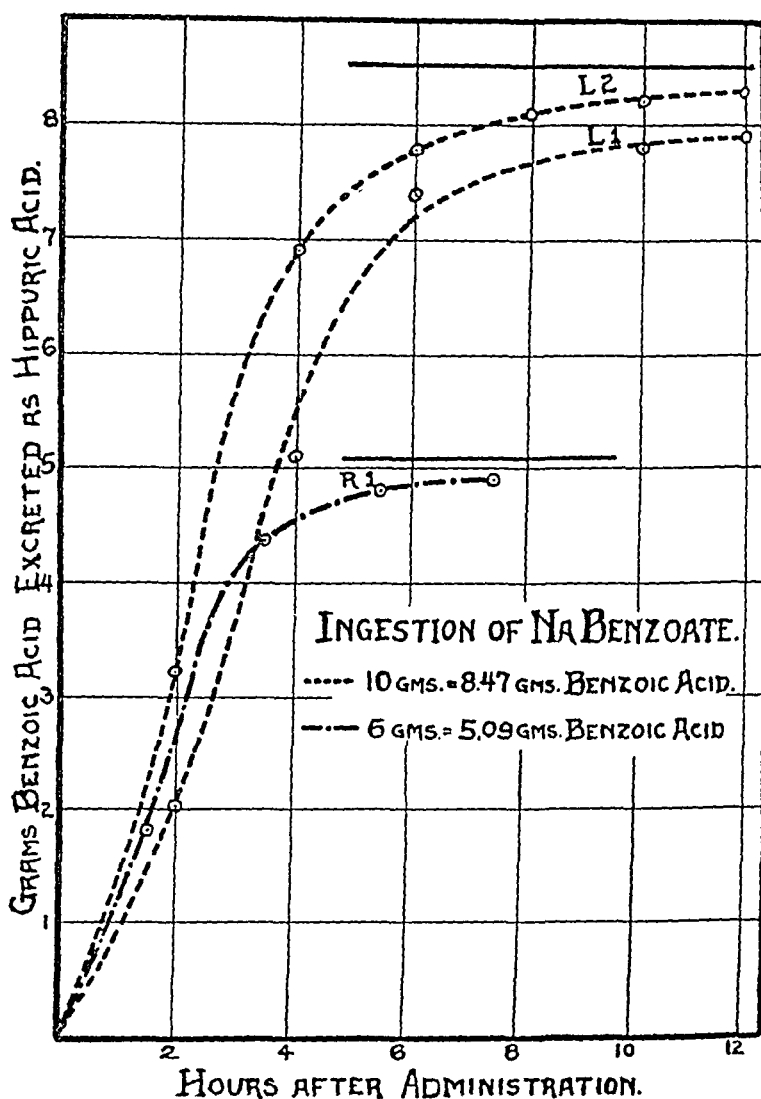
Subject R.

1 hr. 30 min.	1.746	34.3	2.563	Mixed diet, 6 gms. sodium benzoate <i>per os</i> 1 hr. after breakfast.
3 hr. 30 min.	2.599	51.0	3.815	
5 hr. 30 min.	0.464	9.2	0.681	
7 hr. 30 min.	0.140	2.7	0.206	

The rapid synthesis and excretion may best be shown graphically by the accompanying figure, in which the summation curves of the excretion of benzoic acid as hippuric acid in experiments I and II, subject L, and in the experiment with subject R are plotted. The unbroken heavy line in each case represents the amount of benzoic acid fed.

In order to compare the time required for elimination of hippuric acid, with the time required for synthesis and elimination 8.37

grams of sodium hippurate, equivalent to 6 grams of sodium benzoate, were ingested, and the urine collected in periods of one and one-half hours. After one and one-half hours 48.7 per cent of the



hippuric acid fed was recovered in the urine, and after three hours, 81.5 per cent. As was to be expected, the elimination after hippuric acid administration was more rapid than after benzoate ingestion, but the difference is not marked.

It is interesting to compare the results obtained in man with those obtained in rabbits.⁹ Rabbits eliminate hippuric acid within twelve hours after its injection, but after benzoate ingestion there is a delayed elimination, due presumably to slow synthesis, rather than to faulty elimination. The possibility suggests itself that the more rapid elimination in man may be due, in part, to the fact that the kidney, the organ of elimination, is also in man the main organ of synthesis of hippuric acid, and that synthesis and elimination may take place almost simultaneously without the hippuric acid returning to the blood stream. In rabbits, on the other hand, the liver, not the kidney, is the organ primarily concerned with hippuric acid synthesis. It is possible also that the level of nitrogenous metabolism plays a rôle here also. In man, where large amounts of nitrogenous products of metabolism are being constantly set free, the opportunity for the formation of glycocoll, whatever its precursor may be, must be much greater than in the rabbit, into whose circulation much smaller amounts of nitrogenous products enter, in consequence of its lower level of protein metabolism. As a result, nitrogenous material is not available for as rapid a synthesis of hippuric acid in rabbits as in man. Experiments to test these hypotheses are being undertaken.

SUMMARY.

In man after the administration of from 6 to 10 grams of sodium benzoate, elimination as hippuric acid takes place very rapidly, 85 to 90 per cent of the ingested benzoate being excreted in from five to six hours. The rate of elimination of hippuric acid after the administration of sodium benzoate is only slightly less rapid than after the administration of hippuric acid. After benzoate ingestion, the urine collected during the first six hours, the period during which the greater part of the hippuric acid is being excreted, has a lower urea + ammonia content than in a normal control period, indicating that hippuric acid nitrogen is derived at the expense of the nitrogen normally eliminated as urea.

⁹ Raiziss, Raiziss, and Ringer: this *Journal*, xvii, pp. 527-9, 1914.

THE CHEMISTRY OF GLUCONEOGENESIS.

IX. THE FORMATION OF GLUCOSE FROM DIOXYACETONE IN THE DIABETIC ORGANISM.¹

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(Received for publication, May 28, 1914.)

The formation of glucose from dioxyacetone in the perfused liver was demonstrated recently by Embden, Schmitz and Wittenberg.² They found a very marked rise in the glucose concentration of the perfusion fluid after the addition of dioxyacetone.

In these experiments it was our object to study the influence of dioxyacetone on the glucose formation and acidosis in phlorhizinized dogs. We wish to state here that the plans for this work were laid about a year prior to the appearance of Embden's publication.

The dioxyacetone was kindly prepared for us by the Farbwerke-Hoechst vorm. Meister Lucius und Bruning, to whom we take pleasure in expressing our indebtedness.

The methods used were the same as those employed in experiments previously described. Because of the reducing properties of dioxyacetone, the glucose was also determined by means of the polariscopic method and the results given in the tables, so that any reduction of Fehling's solution due to dioxyacetone, which may have been secreted by the kidneys, can be detected.

The G:N ratio calculations are based upon the glucose figures obtained with Allihn's method.

In experiment XXXVIII period VI, 9.0 grams ($\frac{M}{10}$) of dioxyacetone, dissolved in 40 cc. of distilled water, were given subcutaneously. The glucose elimination, which was 7.0 grams in the fore period rose to 13.2 and the G:N ratio, which was 3.02 in the fore period and 3.19 in the after period, rose to 5.64. If we assume that the mean ratio of 3.1 would have obtained nor-

¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² Embden, Schmitz and Wittenberg: *Zeitschr. f. physiol. Chem.*, lxxxviii, p. 210, 1913.

mally in period VI, we find that 5.94 grams of "extra" glucose were eliminated in that period. In this period the acetone and acetoacetic acid were reduced from 200 mgms. to 103 mgms., to rise again to 190 in the after period.

In experiment XXXIX period VI, 9.0 grams of dioxycetone were similarly administered subcutaneously. The glucose elimination, which was 10.9 and 11.66 grams in periods IV and V respectively, rose to 19.2 and 12.96 in periods VI and VII. The G:N ratio in periods V and VIII was 3.15 and 3.04. Assuming the mean ratio of 3.1 for periods VI and VII, we find that 9.88 grams of "extra" glucose were eliminated. In this experiment dioxycetone exercised a fairly strong antiketogenetic effect by causing a drop in the acetone and acetoacetic acid elimination from 227 to 85 mgms., and in the β -hydroxybutyric acid elimination from 910 to 195 mgms.

In experiments XL and XLI the above results are corroborated. The "extra" glucose eliminated in experiment XL was 6.66 grams and in experiment XLI it was 5.3 grams.

In experiment XLI the total carbon³ output in the urine was also studied. The object was to find whether any other carbonaceous material, not utilized in the body, was eliminated as a result of the dioxycetone administration. The carbon of the glucose, acetoacetic acid, β -hydroxybutyric acid was calculated from the figures obtained in their determinations. The sum of these, sub-

TABLE I.
Experiment XLI.

PERIOD	I CARBON IN GLUCOSE	II CARBON IN ACETONE AND ACETO- ACETIC ACID	III CARBON IN β -HYDROXY- BUTYRIC ACID	IV CARBON IN 1 + 2 + 3	V TOTAL CARBON IN URINE	VI UNDETER- MINED CARBON	REMARKS
	1	2	3	α	β	$\beta - \alpha$	
XI	4.87	0.077	0.156	5.10	9.81	4.71	
XII	4.59	0.078	0.137	4.80	9.56	4.76	
XIII	4.98	0.075	0.144	5.20	9.93	4.73	
XIV	7.39	0.068	0.144	7.60	12.16	4.56	{ 9.0 gms. of dioxycetone administered contain- ing 3.6 gms. of carbon.

³ The carbon was determined by the method suggested by Tangl and Kereszty: *Biochem. Zeitschr.*, xxxii, p. 266, 1911.

EXPERIMENT XXXVIII. *Twelve hour periods.*

DATE 1914	PERIOD	WEIGHT	NITROGEN	GLUCOSE (ALUIN)	GLUCOSE (POLARISCOPE)	G: N	"EXTRA" GLUCOSE	ACETONE AND ACETOACETIC ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
Feb.										
24	V		2.32	7.00	6.35	3.02		0.200		9 gms. of dioxycetone dissolved in 40 cc. of water given subcut.
24	VI		2.34	13.20	13.30	5.64	5.94	0.103	0.364	
25	VII		2.17	6.94	6.45	3.19		0.190	0.775	
25	VIII		2.39	5.84	5.20	2.44		0.179	0.642	
26	IX		2.44	5.75	5.20	2.35		0.218	0.503	

EXPERIMENT XXXIX. *Twelve hour periods.*

Feb.										
23	IV		3.41	10.90	10.40	3.20		0.234	0.836	9.0 gms. of dioxycetone as above.
24	V	8.77	3.70	11.66	10.60	3.15		0.227	0.910	
24	VI		3.46	19.20	17.10	5.55	9.88	0.085	0.195	
25	VII	8.53	3.73	12.96	12.50	3.48		0.165	0.416	
25	VIII		3.42	10.40		3.04		0.150	0.435	

EXPERIMENT XL. *Twelve hour periods.*

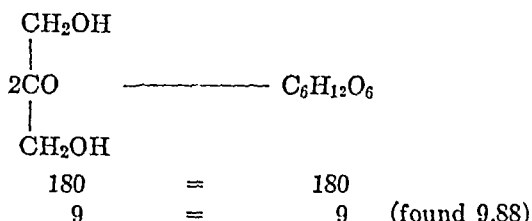
Feb.										
3	II		3.54	10.00		2.82		0.075	0.151	9.0 gms. of dioxycetone dissolved in 50 cc. of water given subcut.
4	III	7.20	3.15	15.70		4.99	6.66	0.046	0.081	
4	IV		3.46	10.10		2.93		0.111	0.238	

EXPERIMENT XLI. *Twelve hour periods.*

May										
8	XI	11.16	4.68	12.19		2.78		0.164	0.339	9.0 gms. of dioxycetone as above.
8	XII		4.39	11.48		2.62		0.167	0.296	
9	XIII		4.48	12.46	11.23	2.78		0.160	0.312	
9	XIV		4.72	18.46	17.65	3.92	5.3	0.145	0.312	
10	XV	10.82				3.20		0.155	0.269	
10	XVI		4.28	12.67	11.86	2.96		0.126	0.273	

tracted from the total carbon, gives the undetermined carbon fraction which would rise if any other product of dioxyacetone metabolism found its way into the urine.

Table I gives the results of this investigation. Column VI gives the values of the undetermined carbon. From the fact that there was no increase in that fraction (period XIV) when dioxyacetone was administered, we feel justified in assuming that all of the dioxyacetone is either converted into glucose or burned. That the conversion of dioxyacetone into glucose may be quantitative is evident from experiment XXXIV.



Miller and Taylor⁴ recently found that dioxyacetone, in acid solution, acts as a very strong reducing agent of ammonium molybdate. This fact was utilized in detecting its presence in the urine in unchanged form. In experiment XL it was strongly positive; the urine also reduced Fehling's solution in the cold. In none of the other experiments was this observed.

SUMMARY AND CONCLUSIONS.

Four experiments were performed in which dioxyacetone was administered subcutaneously to phlorhizinized dogs. In every case a rise in the glucose elimination followed. The "extra" glucose in one experiment corresponded to an amount of glucose which would arise if all of the carbon of dioxyacetone were converted into glucose.

The effect of dioxyacetone on acidosis is decidedly antiketogenic in three of the four experiments.

Dioxyacetone was found in the urine in unchanged form in only one of the four experiments. In that case the urine reduced Fehling's solution in the cold and reacted positively with the Miller-Taylor reagent. In none of the other experiments did dioxyacetone appear in the urine.

⁴ Miller and Taylor: this *Journal*, xvii, p. 531, 1914.

ON THE CONJUGATED SULPHURIC ACID FROM TENDOMUCOID.

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(Received for publication, May 28, 1914.)

The presence of a conjugated sulphuric acid in the molecule of tendomucoid was first discovered by Levene.¹ Later J. A. Mandel and Levene² have shown the presence of a similar acid in many tissues and even in leucocytes. All these new conjugated sulphuric acids resembled in their properties the chondroitin sulphuric acid obtained from cartilage by Mörner³ and by Schmiedeberg.⁴ The exact relationship of these various acids to one another and of all of them towards chondroitin sulphuric acid could not be established with certainty, since the details of the structure of the substance of Mörner and Schmiedeberg had not been known. Provisionally all conjugated sulphuric acids derived from any other source than from cartilage were named glycothionic acids.

In course of the last two years, through the work of the present writers,⁵ our knowledge of the structure of chondroitin sulphuric acid has made considerable progress. All the components of the substance have been isolated and the mode of their union in a great measure was elucidated. One component of chondroitin sulphuric acid was found to be of special significance, namely the amino hexose. This proved to be a new nitrogenous hexose—chondrosamine—quite distinct from glucosamine. The complex

¹ Levene: *Zeitschr. f. physiol. Chem.*, xxxi, p. 395, 1901.

² J. A. Mandel and Levene: *Zeitschr. f. physiol. Chem.*, xlv, p. 336, 1905; *Biochem. Zeitschr.*, iv, p. 78, 1907.

³ Mörner: *Skand. Arch. f. Physiol.*, i, p. 210, 1889.

⁴ Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1903.

⁵ Levene and La Forge: *This Journal*, xv, pp. 69 and 155, 1913; xviii, p. 123, 1914.

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of this hexose with glucuronic acid—chondrosin—is also a very definite component of chondroitin sulphuric acid. Further, the ratio of nitrogen to carbon, 1 : 14, was found quite characteristic for chondroitin sulphuric acid. Finally, the presence of one acetyl group in the molecule may be regarded as typical of the same acid.

The analysis of the conjugated sulphuric acid obtained from tendomucoid resulted in the discovery that the components of this acid were identical with those of chondroitin sulphuric acid: they were the same hexosamine, chondrosamine, the same glucuronic acid, besides acetic and sulphuric acids. Similarly to chondroitin sulphuric acid the substance yielded, on hydrolysis with hydrochloric acid, chondrosin hydrochloride. The ratio of nitrogen to carbon was 1 : 14, as in chondroitin sulphuric acid. The substance similarly to chondroitin sulphuric acid also contained one acetyl group in its molecule.

Hence one is forced to accept the identity of the conjugated sulphuric acid from tendomucoid with that of chondroitin sulphuric acid from cartilage.

EXPERIMENTAL.

Preparation of tendomucoid and chondroitin sulphuric acid.

Owing to the nature of the combined protein the method of preparing chondroitin sulphuric acid from tendons differs somewhat from its preparation from cartilage. Portions of 50 achilles tendons from cattle were cleaned, passed through a hashing machine and allowed to stand over night with 20 liters of two-thirds saturated lime water. The liquid was strained off and the process repeated once again on the residue. The combined filtrates were just acidified with hydrochloric acid which produced a flocculent precipitate of tendomucoid. The supernatant liquid is then siphoned off and after addition of an equal volume of 95 per cent alcohol the mucoid was filtered off on a folded filter. The moist product was agitated for some time with 1.5 liters of a 2 per cent potassium hydrate solution. After standing over night the turbid brown solution was acidified with acetic acid and the separated protein removed by filtration on a folded filter. The filtrates

from two such experiments were neutralized with sodium hydrate and the chondroitin sulphuric acid precipitated by a solution of basic lead acetate. The lead precipitate was repeatedly washed by triturating in a mortar with distilled water and filtering with suction. The washed product was suspended in about 2 liters of water; 10 cc. of glacial acetic acid and 20 grams of barium acetate were added and decomposition effected by passing in hydrogen sulphide with constant stirring. The lead sulphide was filtered off with suction, the filtrate concentrated to about 350 cc., and the barium salt precipitated by the addition of about 250 cc. of alcohol. It was then filtered with suction, washed, first with 50 per cent, then with 95 per cent, and finally with absolute alcohol and ether. The yield amounts to about 12 to 15 grams.

0.2220 gram of substance gave 0.2043 gram CO_2 and 0.0775 gram H_2O .

0.6136 gram of substance gave 9.25 cc. $\frac{N}{10}$ NH_3 (Kjeldahl).

0.6141 gram of substance gave 9.50 cc. $\frac{N}{10}$ acetic acid.

0.7002 gram of substance gave 0.2170 gram BaSO_4 .

	Calculated for $\text{C}_{22}\text{H}_{44}\text{N}_2\text{S}_2\text{O}_{22}$:	Found:
C.....	27.80	25.13
H.....	3.48	3.88
N.....	2.32	2.11
S.....	5.30	4.26
Ba.....	22.70	18.35
N : C = 1 : 13.89		

Chondrosin hydrochloride was prepared exactly as described in a previous communication.⁶

0.1258 gram of substance gave 0.1653 gram CO_2 and 0.0685 gram H_2O .

0.2238 gram of substance gave 13.6 cc. amino N at 20° , 763 mm.

0.4024 gram of substance in 3 cc. of water, weight of solution 3.3792 grams, rotated in a 0.5 dm. tube at 20° with D-light $+2.46^\circ$.

Chondrosin hydrochloride from cartilage.

0.1584 gram of substance gave 0.2167 gram CO_2 and 0.0806 gram H_2O .

0.2085 gram of substance gave 12.5 cc. amino N at 18° , 765 mm.

0.6543 gram of substance in 3 cc. of water, weight of solution 3.6481 grams, rotated in a 0.5 dm. tube with D-light $+3.90^\circ$.

⁶ This *Journal*, xv, p. 73, 1913.

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	Calculated for $C_{12}H_{21}NO_{11}HCl$ (390.5):	From tendons	Found: From cartilage
C.....	36.9	35.8	37.3
H.....	5.64	6.0	5.66
N.....	3.58	3.45	3.45
N : C =		1:12.1	1:12.6
$[\alpha]_D^{20}$		+41.5	+43.4

The *hexosamine hydrochloride* was prepared in exactly the same manner as already described.⁷ From 18 grams of the barium salt 3.5 grams amino hexose were obtained. For analysis it was dissolved in 3 parts of water with the addition of a few drops of hydrochloric acid, allowed to crystallize by evaporation and dried in a desiccator. M.P., 180°.

0.1596 gram of substance gave 0.1930 gram CO_2 and 0.0945 gram H_2O .

0.1516 gram of substance gave 17.6 cc. amino N at 19°, 774 mm.

0.1932 gram of substance in 2 cc. of water, weight of solution 2.1904 grams, rotated in a 1 dm. tube at 20° with D-light:

After about fifteen minutes..... +10.75°

After twenty-four hours..... + 8.5°

	Calculated for $C_6H_{13}O_5NHCl$:	Found:
C.....	33.40	33.01
H.....	6.54	6.57
N.....	6.51	6.79
$[\alpha]_D^{20}$ (for equilibrium condition without consideration of specific gravity) = +96.4°.		

Glucuronic acid osazone hydrazid was prepared exactly as previously described.⁸ From 4 grams of chondrosin hydrochloride 0.1 gram of the substance was obtained; M.P. exactly the same as the product from glucuronic acid cartilage chondrosin, 122°.⁹

0.0805 gram of substance gave 12.1 cc. N (Dumas) at 24°, 766 mm.

	Calculated for $C_3H_3N_4O_4 + 1.5 H_2O$:	Found:
N.....	17.17	16.93

⁷ This *Journal*, xv, p. 158, 1913; xviii, p. 123, 1914.

⁸ This *Journal*, xv, p. 75, 1913.

⁹ This melting point, owing to a typographical error, was reported 115° (this *Journal*, xv, p. 75). The correct melting point is 122° with decomposition.

THE METABOLIC RELATIONSHIPS OF THE ACETONE SUBSTANCES.

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(Received for publication, May 30, 1914.)

Considerable uncertainty still exists as to just what relation acetoacetic acid and oxybutyric acid bear to each other and to the fatty acid chain in the catabolic processes. In the present investigation an attempt has been made to clear up some of this uncertainty and, in particular, to answer the following questions:

1. In the conversion of fatty acids into oxybutyric acid and acetoacetic acid, which of these substances is to be considered the final and which the intermediate stage?

2. Can the organism effect the transformation of oxybutyric acid to acetoacetic acid and vice versa, and if so, in what direction does the reaction proceed most readily?

3. What is the explanation for the appearance of only one of the isomers of oxybutyric acid in the urine, namely levorotatory oxybutyric acid?

In the fairly extensive literature bearing on the subject of the metabolism of the acetone bodies these questions have already been considered in some detail and answers proposed, but the answers of different investigators have not been in accord.

In a comprehensive monograph,¹ Dakin has reviewed the literature on this subject up to the year 1912. Since that time, the most noteworthy investigations bearing on the particular questions considered here have been the perfusion experiments of Embden and the perfusion and injection experiments of Friedmann and their collaborators.

¹ Dakin: *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company.

Taking all experimental work into consideration it may be stated that the prevailing opinion has been that the catabolism of fatty acids is accomplished by successive β -oxidation resulting in the production of butyric acid which is then converted into oxybutyric acid and this to aceto-acetic acid, which latter substance is presumably converted into carbon dioxide and water by passing through the stage of acetic acid. This view has been questioned by Blum² who maintains, from the results of his injection experiments, that normally butyric acid is first converted into acetoacetic acid and that this is reduced to oxybutyric acid. That acetoacetic acid may be reduced to *l*-oxybutyric acid has indeed been demonstrated by the liver perfusion experiments of Friedmann and Maase,³ and the incubated hashed liver and injection experiments of Dakin,⁴ and of Langermark,⁵ and this fact would appear to confirm Blum's view. Dakin has also demonstrated the presence in the liver of another enzyme capable of producing the reverse change, namely oxidation of oxybutyric acid to acetoacetic acid. Dakin mentions, however,⁶ that "under ordinary conditions a normal minced dog's liver is more active in reducing acetoacetic acid than in oxidizing hydroxybutyric acid," but adds that "these observations are of little value in judging of the reactions during life." I have confirmed these observations of Dakin and have found that the intact living organism behaves, in this particular, similarly to the hashed livers.

On the basis of his incubation experiments,⁷ Dakin has suggested that acetoacetic acid is asymmetrically reduced, in the body, with the formation of *l*-oxybutyric acid and explains the excretion of an excess of *l*-oxybutyric acid following the injection of *dl*-oxybutyric acid as due to an oxidation of this substance to aceto-acetic acid and subsequent asymmetric reduction. This hypothesis fails to account for some experimental findings which will be referred to later in this paper.

The work referred to above concerns primarily the acetone bodies

² Blum: *Münch. med. Wochenschr.*, lvii, p. 683, 1910.

³ Friedmann and Maase: *Biochem. Zeitschr.*, xxvii, p. 474, 1910.

⁴ Dakin: *This Journal*, viii, p. 97, 1910.

⁵ Langermark: *Biochem. Zeitschr.*, lv, p. 458, 1913.

⁶ Dakin: *Oxidations and Reductions in the Animal Body*, p. 25.

⁷ Dakin: *This Journal*, viii, p. 102, 1910.

as products of fatty acid catabolism, and it is this phase of the subject which will be considered in this paper, since acetone bodies produced in the catabolism of amino-acids, once they are formed, would be expected to behave the same way in the organism as those derived from fatty acids.

The recent observations of Loeb⁸ and Embden and Loeb⁹ and of Friedmann¹⁰ on the formation of acetone bodies by synthesis from acetic acid on perfusion through glycogen-free livers are interesting but until we have further information as to the source of acetic acid in the normal metabolism, these results can hardly be said to bear on the particular subjects under consideration.

I have repeated some of the experiments of other workers referred to above. I have incubated fresh hashed organs with the salts of acetoacetic acid and of oxybutyric acid, and have injected these acids into normal and phlorhizinized dogs and into normal fasting pigs. My work, however, differs from that of other observers in that simultaneous separate determinations of acetoacetic acid and of oxybutyric acid were made in all cases, thus enabling a comparison to be made of the amount of each converted into the other, as well as the amounts otherwise destroyed. Until recently, in most laboratories, the determination of oxybutyric acid has been accomplished by means of its optical activity. This method, while satisfactory enough when applied to urines containing much oxybutyric acid, is of very questionable accuracy when applied to the analysis of blood and organs in which vanishingly small amounts of oxybutyric acid are present, while at the same time other optically active substances, some of them soluble in ether, also occur. A more suitable method for blood and tissue analysis is the procedure based upon the bichromate oxidation method of Shaffer, the details of which I have already described.¹¹ This method assuredly gives more nearly correct values under the conditions, and has been the method used throughout the present investigation. Parallel determinations of oxybutyric acid by the optical method were also made in some instances.

In a number of experiments the circulating blood of intact

⁸ Loeb: *Biochem. Zeitschr.*, xlvii, p. 118, 1912.

⁹ Embden and Loeb: *Zeitschr. f. physiol. Chem.*, lxxxviii, p. 246, 1913.

¹⁰ Friedmann: *Biochem. Zeitschr.*, lv, p. 436, 1913.

¹¹ Marriott: *This Journal*, xvi, p. 293, 1913.

animals was analyzed at intervals for acetoacetic acid and oxybutyric acid. This is a method that has not previously been applied to this problem and one which has been shown to be capable of indicating more precisely the intermediate metabolic transformations of the acetone substances than the usual urinary analyses. As only about 2 cc. of blood were required for an analysis, there was no interference with the condition of the animal.

The fate of oxybutyric acid.

Salts of *dl*-oxybutyric acid were incubated for several hours with fresh hashed portions of dogs' muscle, and liver, and with blood, under conditions favorable to oxidation processes, with the result that but small amounts of oxybutyric acid disappeared, and, at the same time, practically no acetoacetic acid or acetone was produced (Experiments 1-2). The oxybutyric acid was apparently not, to any extent, converted into acetoacetic acid, but was destroyed in some other way.

On injecting oxybutyric acid into living animals, similar results were obtained. The intravenous injection of *dl*-sodium oxybutyrate (Experiment 4) equivalent to 3.63 grams acetone, into a small fasting dog was almost entirely destroyed within eighty minutes time, only 10 per cent being recoverable from the urine, liver and blood. At the same time less than 50 mgm. were present in the form of acetone and acetoacetic acid, altogether, in the urine, liver and blood. No acetone could be detected in the breath. Such acetoacetic acid as was present, was, in proportion to the oxybutyric acid, more abundant in the blood than elsewhere. The urine was extracted and examined for optical activity by the method of Black¹² and was found to be very slightly dextrorotatory. Extracts of the liver and blood were optically inactive. There was no evidence, therefore, that an excess of *l*-oxybutyric acid was present.

For some of the injection experiments suckling pigs were used, as it has been claimed by Baer¹³ that these animals are especially prone to the development of an acidosis. Synthetic *dl*-sodium

¹² Black: *This Journal*, v, p. 209, 1908.

¹³ Baer: *Arch. f. exp. Path.*, liv, p. 153, 1906.

oxybutyrate equivalent to 4 grams of acetone was injected subcutaneously into a 4.5-kilo. pig (Experiment 5). Blood samples were analyzed at intervals. The oxybutyric acid content of the blood rose very quickly, as was to have been expected. At the same time, there was a small increase of acetoacetic acid present in the blood, but the amount was only about one-tenth as great as that of oxybutyric acid present.

The intravenous injections of considerable quantities of *dl*-sodium oxybutyrate (Experiment 8), as well as of *l*-sodium oxybutyrate (Experiment 7) into phlorhizinized dogs caused the excretion of but insignificant amounts of acetoacetic acid in the urine, and in this particular, no differences were noted between the behavior of the *dl* and the *l* salts. Considerable difference was noted, however, in the character and quantity of the oxybutyric acid excreted in the urine following the injection of the optically isomeric oxybutyric acids. Experiments were performed on three dogs each of which received in one experiment (Experiment 7) *l*-sodium oxybutyrate equivalent to 3.54 grams of acetone, and in a second experiment (Experiment 8) an equivalent amount of *dl*-sodium oxybutyrate.

On injection of the levo salt, the animals showed individual variations (Table V). Dog A excreted unchanged all of the injected oxybutyric acid, whereas dogs B and C excreted respectively 82 per cent and 50 per cent. In calculating the amounts of oxybutyric acid excreted due to the injection it was, of course, necessary to subtract the amount the animal was already passing in the urine. To this end, the figures for the fore day and after day were averaged.

Determinations of the oxybutyric acid of these urines by the optical method, according to Black's procedure, gave figures agreeing with those obtained by the oxidation method, indicating, as was to have been expected, that following the injection of levo acid all of the acid excreted was of the levo variety.

In case of the urines passed following injections of *dl*-oxybutyric acid (Table VI), the figures obtained by the oxidation method and by the Black optical method were quite different, as is seen from the following tabulation.

Dog A: October 17	{	Oxybutyric acid by oxidation.....	grams 4.02 ¹⁴
		Oxybutyric acid by optical activity..	2.52
Dog B: October 17	{	Oxybutyric acid by oxidation.....	4.02
		Oxybutyric acid by optical activity..	2.30
Dog C: October 17	{	Oxybutyric acid by oxidation.....	2.08
		Oxybutyric acid by optical activity..	0.75

From the figures just given it is possible to calculate how much of the excreted oxybutyric acid was of the levo form and how much of the dextro form. For example dog A received *dl*-oxybutyric acid equivalent to 3.54 grams acetone, or 1.77 grams each of the *d* and *l* forms. The animal was excreting some levo acid before the injection and continued to do so on the second day following. Taking the average of the figures for the 16th (1.00 gram) and the 18th (2.10 grams), we have 1.55 grams, or the amount of levo acid which we would expect to have been excreted on the 17th, had no injection been made. Any oxybutyric acid in excess of this figure can be assumed to be the result of the injection. A total of 4.02 grams was excreted which had an optical activity corresponding to 2.52 grams of levo acid, the remainder or 1.50 grams must have been *dl* acid, or 0.75 gram of *d* acid and 0.75 gram of *l* acid. Adding this amount of *l* acid to the 2.52 grams we have a total of 3.27 grams of *l* acid excreted as compared with 0.75 gram of the *d* acid. From the *l* acid we must subtract the amount the animal would have excreted, had no injection been made, namely 1.55 grams. As a final result, we have excreted 1.72 grams of *l* acid and 0.75 grams of *d* acid, following the injection of 1.77 grams of *l* acid and 1.77 grams of *d* acid. Although all of the *l* acid injected was excreted, a large part of the *d* acid was retained and presumably burned. It does not seem likely that it was converted into *l* acid by passing through the stage of acetoacetic acid, according to the ingenious hypothesis of Dakin.¹⁵ For if this were true, we should expect that in an animal already shown to be incapable of burning *l* acid (Experiment 8), there would be a much higher excretion of this substance, and furthermore, that the acetoacetic output would be increased materially.

Applying the same methods of calculation to the results obtained from dogs B and C, we obtain the following figures. Each dog

¹⁴ Results expressed in terms of acetone.

¹⁵ Dakin: *Loc cit.*

received the equivalent of 1.77 grams of *l* acid and 1.77 grams of *d* acid, and as a result dog B excreted 1.55 gram of *l* and 0.86 gram of *d* acid. Dog C excreted 1.15 grams of *l* acid and 0.67 gram of *d* acid.

It is interesting to note that each animal excreted very nearly the same proportion of injected *l* acid as in Experiment 7, in which only *l* acid was injected. In actual figures dog A excreted 90 per cent, dog B 87 per cent, and dog C 65 per cent of the injected levo acid.

From such experiments it seems probable that in the phlorhizinized organism, at least, *dextrorotatory oxybutyric acid can be burned with considerable ease, but that to the contrary, the levorotatory isomer cannot be readily burned.* As a corollary, it would follow that for each molecule of levo oxybutyric acid excreted, an equivalent amount of dextro acid must have been burned in the body, or at least, changed to some other substance.

All of the experiments with oxybutyric acid indicate that *oxybutyric acid is with difficulty, if at all, converted into acetoacetic acid in the organism.*

The fate of acetoacetic acid.

On incubating solutions of sodium acetoacetate with fresh liver and muscle hashes (Experiment 3), there was a disappearance of acetoacetic acid and a simultaneous appearance of approximately one-half the equivalent amount of oxybutyric acid. Portions of the same liver and muscle, on incubation with an equivalent amount of oxybutyric acid, caused the disappearance of only about a tenth as much of the added oxybutyric acid. This was quite in accord with the experiment previously described. It appears that *fresh organ hashes can accomplish the transformation of acetoacetic acid far more readily than that of oxybutyric acid.* From the optical activity of the extracts of the livers used in these experiments most of the oxybutyric acid formed from acetoacetic acid was of the *l* rotatory modification.

The injection of sodium acetoacetate, subcutaneously, into a suckling pig (Experiment 5) led to interesting results (Table III). The acetoacetic acid of the blood rose to a moderate extent, but there was also a rise of the oxybutyric acid to such an extent that there was actually more oxybutyric acid present than acetoacetic.

This rather astonishing result is further indication that acetoacetic acid is, in the organism, readily converted into oxybutyric acid. A comparison with the figures obtained on injection of an equivalent amount of oxybutyric acid given in a parallel column (Table III) shows that the reverse change, that is transformation of oxybutyric acid to acetoacetic acid is accomplished with considerable difficulty.

Sodium acetoacetate solutions were also injected into phlorrhizinized dogs (Experiment 6). The animals were the same as those used for the oxybutyric acid injections above described (Experiments 7 and 8). The animals showed individual differences, but it was evident that, in all of them (Table IV), a considerable portion of the injected acetoacetic acid was excreted in the form of oxybutyric acid, the quantity of this latter being (as determined by the Shaffer method) in the case of dogs A and B almost two-thirds as much as that of the unchanged acetoacetic acid eliminated, and in the case of dog C one-half. When we consider that a large amount of the injected acetoacetic acid was probably eliminated almost at once by the kidneys before it could be considered as having entered the tissues, the readiness with which the conversion into oxybutyric acid takes place is the more apparent. In order to determine the optical properties of the oxybutyric acid formed, the urines were made alkaline, evaporated to small volume, acidified, set with plaster and extracted with ether according to the Black procedure. The extracts were evaporated, taken up with water and examined in the polariscope. The same extracts were then used for another determination by the Shaffer method with the following results expressed in terms of acetone and calculated for the whole volume of urine:

		grams
Dog A: Oct. 7	Oxybutyric acid by oxidation.....	1.95
	<i>l</i> -Oxybutyric acid by optical value	1.80
	Oxybutyric acid by oxidation of polarized extract.....	2.00
Dog B: Oct. 7	Oxybutyric acid by oxidation.....	1.95
	<i>l</i> -Oxybutyric acid by optical value.....	1.46
	Oxybutyric acid by oxidation of polarized extract.....	1.89
Dog C: Oct. 7	Oxybutyric acid by oxidation.....	0.96
	<i>l</i> -Oxybutyric acid by optical value.....	0.61
	Oxybutyric acid by oxidation of polarized extract.....	1.09

The figures obtained by the oxidation procedure indicate more oxybutyric acid than can be accounted for by the optical value. As the distillates from the bichromate oxidation were all redistilled from alkali and peroxide, it is hardly possible that the excess was due to substances other than oxybutyric acid. Neither lactic acid, phlorhizin nor sugar could be detected in the final extracts that were used for polarization. The results would seem to indicate that *a small portion of the excreted oxybutyric acid was of the dextrorotatory modification.*

To further test this point a small dog was fasted, phlorhizinized, and injected daily with solutions of sodium acetoacetate in the same way as just described. The urines were collected and oxybutyric acid extracted by the Black method and converted into the calcium zinc double salt as described by Shaffer and Marriott.¹⁶ The salt recrystallized once from alcohol and once from water, was dried in a desiccator over calcium chloride. A 7 per cent solution of this salt was found to have a specific rotation of -14.82° , as against -16.26° for the pure levo salt, thus indicating the presence of some dextrorotatory component. As the levo salt is somewhat less soluble than the racemic, we would expect further recrystallization to give a salt with less of the racemic constituent; so that the amount of dextro salt present originally in the urine was probably even greater than would be indicated by the results obtained after two crystallizations.

If a part of the injected acetoacetic acid is converted into dextrorotatory oxybutyric acid and a part into the levo rotatory form, we would expect that the two forms must be produced in equal amounts and the appearance of a large excess of one form in the urine must be an indication that the other form is burned or converted into some optically inactive substance in the organism. If this were true we would expect acetoacetic acid, on injection into animals, or incubation with organ hashes, to give rise to only one-half the theoretically obtainable oxybutyric acid, and this is, in fact, exactly what has been found to take place. Mention of this fact has already been made above in discussing the incubation experiments (Experiment 3). The results of Friedmann and Maase¹⁷ are also entirely in accord with mine on this point.

¹⁶ Shaffer and Marriott: *This Journal*, xvi, p. 265, 1913.

¹⁷ Friedmann and Maase: *Biochem. Zeitschr.*, xxvii, p. 474, 1910.

On injection of acetoacetic acid into phlorhizinized dogs (Experiment 6) approximately one-half as much *l*-oxybutyric acid was found as could be accounted for by the acetoacetic acid disappearing.

The experiments with acetoacetic acid indicate that it is quite readily converted into oxybutyric acid in the organism and it appears probable that equal amounts of d- and l-oxybutyric acid are formed and, of the isomeric oxybutyric acids thus produced, the d form is the more easily destroyed.

Considering together the results of the experiments with the oxybutyric acids and with acetoacetic acid, it would appear that although, in the organism, they are convertible the one into the other, the preponderating reaction is that of transformation of acetoacetic acid into oxybutyric acid, the aceto-acetic acid being but an intermediate step between the fatty acids, on the one hand, and oxybutyric acid on the other. That is to say, the path of fatty acid catabolism is as follows: Fatty acid—→ Acetoacetic acid—→ Oxybutyric acid. Hence failure to burn oxybutyric acid would cause an accumulation of acetoacetic acid in a similar way that the end products of enzymatic reactions, when in sufficient concentration, usually cause an accumulation of intermediate products.¹⁸ This may be the explanation for the small rises in acetoacetic acid following oxybutyric acid injection, or following the addition of oxybutyric acid to hashed organs. It is possible that under certain circumstances a small amount of oxybutyric acid may be converted into acetoacetic acid, but apparently this is again changed back to oxybutyric acid before it is burned. In other words, *acetoacetic acid is normally catabolized by passing through the oxybutyric acid stage.*

Further confirmation of this conception of fatty acid catabolism is furnished by an experiment (Experiment 9) in which sodium butyrate was injected into a fasting dog. The results of analyses of blood, taken at intervals (Table VII), indicate that butyric acid, on injection, behaves almost exactly like acetoacetic acid and quite differently from oxybutyric acid. There was first a rise of the acetoacetic acid of the blood, and this was followed by a rise in oxybutyric acid. This would lead one to suppose that butyric

¹⁸ This suggestion has previously been made by Neubauer: *Verhandlung d. xxvii, Kong. Inn. Med.*, p. 566, 1910.

acid is, in the body, first converted into acetoacetic acid, and that oxybutyric acid is only formed secondarily.

We are, however, still in the dark as to the way in which oxybutyric acid is catabolized. Apparently, it does not pass over to acetoacetic acid but is burned in some other way. The hypothesis recently advanced by Ringer¹⁹ may throw some light on the question. Ringer supposes that oxybutyric acid may be burned by first uniting with glucose to form a glucoside-like compound which subsequently breaks down to form non-ketogenic compounds, but that the diabetic organism is incapable of effecting this glucoside union, as well as other glucoside unions, such as the condensation of glucose to maltose and glycogen. This hypothesis, however, as it stands, gives no explanation of the failure of the diabetic to utilize *l*-oxybutyric acid while it appears to utilize the *d* form completely.

Perhaps the key to the situation may be found in the two tautomeric forms of glucose, the α form and the β , capable of forming respectively the α and β series of glucosides. Freshly prepared solutions of glucose contain an excess of the α form, which is gradually in part converted into the β form, equilibrium being finally attained. Dilute solutions contain relatively an excess of the β form,²⁰ and the presence of small amounts of alkali favors the formation of the β tautomer.²¹ It is presumable, then, that in the blood the greater part, at least, of the glucose present is in the β form.

Even the diabetic organism would contain glucose in this form, and we may suppose, following Ringer's idea, that it is used to effect the combustion of *d*-oxybutyric acid. For the burning of *l*-oxybutyric acid, however, a union with α -glucose may be necessary and the diabetic organism may be lacking in this form of glucose. The normal organism, however, is possessed of a mechanism whereby α -glucose is being continually produced, namely the formation and subsequent hydrolysis of glycogen, which is known to be a compound α -glucose-glucoside.²² In the body the transformation of glycogen into glucose occurs, according to the present

¹⁹ Ringer: *This Journal*, xvii, p. 107, 1914.

²⁰ Tauret, quoted by Von Lipmann: *Chemie der Zuckerarten*, 3 ed., p. 279.

²¹ Svoboda: *Zeit. d. Vereins d. d. Zuckerind.*, xlv, p. 107, 1896.

²² Glycogen on hydrolysis gives maltose which latter has been shown to be an α -glucoside by its fermentation reactions (Fischer: *Ber. d. d. chem. Gesell.*, xxvii, p. 2985, 1894).

accepted view, chiefly in the liver, and to some extent, in the muscles. As it is in just these organs that the greatest formation of oxybutyric acid has been shown to occur,²³ the conditions for the formation of a glucoside union are favorable. From this, it is not to be inferred that glucose is burned only by uniting with oxybutyric acid, though it would seem likely that it may unite with other substances to form glucoside-like combinations, and particularly the α -glucoside type. So far as we know, the conversion of glucose into this α form can be accomplished, in the body, only by passing through the glycogen or at least the maltose stage. In diabetes we recognize a serious disturbance in the glycogen forming power of the organism. With failure to form glycogen there is diminished power to burn glucose and oxybutyric acid.

That passage through the glycogen stage alters the character of glucose as far as its antiketoplastic activity is concerned is well illustrated in the experiments of Embden and Wirth,²⁴ in which a substance (isovaleric acid) which formed large amounts of acetone bodies on perfusion through the glycogen-poor livers of starving dogs, failed to give acetone bodies in any great extent on perfusion through glycogen-rich livers. The addition of glucose²⁵ to the perfusion fluid used in the case of glycogen-poor livers had no appreciable effect in inhibiting acetone-body formation. At the same time it did not increase the glycogen content of these livers. Glucose generated from the hydrolysis of glycogen seems to act differently in the organism from glucose derived from other sources, and the most reasonable explanation of this difference in behavior would seem to be the formation of α -glucose. It is not, of course, supposed that this form of glucose would be thrown into the circulation and exist as such for any length of time, nor is such an assumption at all necessary to explain the observed facts. What is necessary, however, according to this hypothesis, is that the unstable α -glucose shall be produced and be present in the tissues involved in oxybutyric acid catabolism. The subcutaneous or intraperitoneal injection of glycogen²⁶ does not suffice for this purpose, for,

²³ Langermark: *Biochem. Zeitschr.*, lv., p. 458, 1913.

²⁴ Embden and Wirth: *Biochem. Zeitschr.*, xxvii, p. 1, 1910.

²⁵ The glucose added in dilute solution would presumably contain mostly α -glucose.

²⁶ Cook: *Proc. Soc. of Exper. Biol. and Med.*, x, p. 39, 1912.

before absorption, the glycogen is presumably hydrolyzed and the net result is the same as if carbohydrate were fed by mouth to the diabetic.

It may be argued that if *d*-oxybutyric acid is burned in the body by uniting with α -glucose, then considerable glucose must be burned at the same time. This does not necessarily follow, as the glucoside union with oxybutyric acid would give rise, according to Ringer, to glucose but not to the acetone bodies. The conception outlined above is of course almost wholly hypothetical, but it appears to account for a number of the observed facts and is, so far as I know, not contradicted by other facts.

EXPERIMENTAL PART.

Experiments on incubated hashed organs and blood.

Dogs, previously starved for from eighteen to twenty-four hours, were anaesthetized by morphine and ether and bled from the carotid artery. The blood was collected in sterile flasks containing a few glass beads; defibrinated by shaking and filtered through glass wool into bottles for incubation. The liver and portions of muscles from the hind legs were quickly removed, hashed in a warmed machine, and immediately transferred to warmed glass-stoppered bottles with the addition of saline solution and added materials, and placed in the incubator. All instruments and receptacles had been previously sterilized by steam. An effort was made to prevent organs from becoming chilled much below body temperature during the processes of transference and chopping.

After incubation, the bottles were cooled in running water, before opening, in order to avoid loss of any acetone which might be present. The contents of the bottles were then analyzed for acetoacetic acid and oxybutyric acid according to the methods described in a previous communication.²⁷ The results throughout this work are expressed in terms of acetone obtained from acetoacetic acid on the one hand, and from oxybutyric acid on the other, and not as acetoacetic acid or oxybutyric acid as such.

Incubation with dl-oxybutyric acid. EXPERIMENT 1. Dog weighing 15 kgm., starved eighteen hours. Four 50-gram portions each

²⁷ Marriott: *This Journal*, xvi, p. 293, 1913.

of blood, liver, and muscle obtained as described above, were placed in 1000 cc. bottles together with 250 cc. of normal saline solution. To two of the four samples of each organ were added 10 cc. of a solution of optically inactive sodium oxybutyrate,²⁸ equivalent to approximately 100 mgm. of acetone. One portion of each organ with, and one portion without, the addition of the oxybutyrate solution, were analyzed at once. The other two portions of each organ were incubated five hours, and then subjected to analysis. Table I gives the results expressed in milligrams of acetone per 50 grams of organ or blood.

EXPERIMENT 2. Dog weighing 14 kgm., starved twenty-four hours, was bled and the liver removed, hashed and divided into four 70-gram portions. To two of the portions added 50 cc. of a solution of optically inactive ammonium oxybutyrate equivalent to approximately 200 mgm. of acetone. Two of the samples, one containing added oxybutyrate and the other none, were incubated in 2000 cc. bottles for five hours, with frequent shaking. The other two portions were analyzed at once. The results appear in Table I.

Incubation with acetoacetic acid. EXPERIMENT 3. Dog weighing 12 kgm. starved twenty-four hours, was bled and liver and muscles hashed. 50-gram portions of liver and of muscle were placed in 200 cc. glass stoppered bottles, together with 50 cc. of a freshly prepared solution of sodium acetoacetate²⁹ equivalent to approximately 200 mgm. of acetone. The bottles were incubated five hours. Two other portions of liver and muscle were analyzed at once. The results appear in Table I.

Blood and liver of this same animal when incubated with solutions of oxybutyrates in large bottles caused the disappearance of but small quantities of oxybutyric acid as is seen in the table.

²⁸ Prepared by the method of Wislicenus: *Ann. d. Chem.*, cxlix, p. 205, 1869.

²⁹ Prepared according to Embden and Michaud: *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 332, 1908.

TABLE I.

Incubation of organs with and without the addition of the salts of acetoacetic acid and of oxybutyric acid. Incubation time 5 hours. Oxybutyric acid and acetoacetic acid expressed in terms of acetone.

EXPERIMENT NUMBER	ORGANS USED	ADDED MATERIAL	ACETONE AND ACETO- ACETIC ACID			OXYBUTYRIC ACID		
			Before incu- bation	After incu- bation	Differ- ence	Before incu- bation	After incu- bation	Differ- ence
			mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
1	50 gm. blood..	250 cc. normal saline.	0.0	0.0	± 0.0	4.8	2.5	-2.3
	50 gm. muscle	250 cc. normal saline.	0.2	0.2	± 0.0	6.3	3.6	-2.7
	50 gm. liver...	250 cc. normal saline.	0.1	0.4	+0.3	9.2	5.8	-3.4
	50 gm. blood..	250 cc. normal saline plus approx. 100 mg of <i>dl</i> -sodium oxybu- tyrate.....	0.0	0.0	+0.0	100.0	92.5	-7.5
	50 gm. muscle	250 cc. normal saline plus approx. 100 mg. of <i>dl</i> -sodium oxybu- tyrate.....	0.4	0.6	+0.2	101.2	99.4	-1.8
	50 gm. liver...	250 cc. normal saline plus approx. 100 mg. of <i>dl</i> sodium oxybu- tyrate.....	0.3	1.0	+0.7	103.6	93.8	-9.8
	70 gm. liver...	250 cc. normal saline plus approx. 200 mg. of <i>dl</i> -ammonium oxybutyrate.....	1.8	4.8	+3.0	187.2	182.1	-5.1
	70 gm. liver...	250 cc. normal saline.	0.5	0.6	+0.1	6.5	6.9	-0.6
	50 gm. liver...	50 cc. solution con- taining approx. 200 mg. of sodium ace- toacetate.....	208.0	156.0	-52.0	6.2	37.6	+31.4
	50 gm. muscle	50 cc. solution con- taining approx. 200 mg. of sodium ace- toacetate.....	214.0	152.0	-62.0	2.1	21.1	+19.1
3	50 gm. liver...	50 cc. solution con- taining approx. 200 mg. of sodium oxy- butyrate.....	1.4	4.7	+3.3	183.5	176.2	-7.3
	50 gm. muscle	50 cc. solution con- taining approx. 200 mg. of sodium oxy- butyrate.....	0.4	4.8	+4.4	185.5	182.8	-2.7

Experiments on the normal intact animal.

EXPERIMENT 4. A dog weighing 9.4 kgm., after twenty-four hours' fast was anaesthetized with ether. A tracheal cannula was inserted and artificial respiration used at intervals. The animal was catheterized and the catheter allowed to remain in the bladder. 250 cc. of a solution containing optically inactive sodium oxybutyrate, equivalent to 3.63 grams of acetone, were run into the femoral vein through a cannula. The solution was warmed to 38°C. The time required to run in all of the material was 40 minutes. At the end of one hour and twenty minutes from the beginning of the experiment the animal was bled from the carotid. The blood was collected, defibrinated and analyzed as in the previous experiments. The liver, weighing 350 grams, was removed, hashed and analyzed, as were also portions of muscle tissue. The bladder was washed and the washings added to the urine passed during the experiment. Urine volume, 112 cc.

Preformed acetone was determined in the urine and in the blood, muscle, and liver by the method of Embden and Schliepp,³⁰ and acetoacetic acid and oxybutyric acid as in the preceding experiments.

An effort was made to determine the acetone in the breath. Valves in the tracheal cannula were so arranged that on inspiration fresh air was drawn into the lungs. The expired air all passed into a rubber balloon of about three liters capacity, and thence through two wash bottles containing alkali to which had been added, just at the beginning of the experiment, a known amount of standard iodine solution. The wash bottles were kept cool with cracked ice. The expired air was passed through the wash bottles for thirty minutes during the time the oxybutyric acid solution was being run in. Forty minutes later fresh hypoiodite was introduced into the wash bottles and the expired air passed through for another thirty-minute period. At the end of each respiration period the contents of the wash bottles were acidified and titrated with standard thiosulphate in the usual way. The results showed that no acetone at all was present in the expired air during either respira-

³⁰ Embden and Schliepp: *Zentralbl. f. d. ges. Physiol. u. Pathol. d. Stoffwechsels*, (N. F.), ii, p. 250, and p. 289, 1907.

tion period.³¹ From this it would seem unlikely that any acetone was eliminated through the lungs during the entire experiment.

The results of the analyses of the urine and of the individual organs appear in Table III. They are expressed in terms of milligrams of acetone. The calculation of the total blood volume was made from the figures of Haldane and Smith.³²

TABLE II.

Experiment 4. Analysis of the organs of a dog eighty minutes after the injection of a solution of dl-sodium oxybutyrate equivalent to 3.63 grams of acetone. Results for acetoacetic acid and oxybutyric acid expressed in terms of acetone.

	BLOOD 100 GMS.	LIVER 100 GMS.	MUSCLE 100 GMS.	BLOOD TOTAL	LIVER TOTAL	URINE TOTAL
Preformed acetone, mgm.	0.3	0.3	0.0	1.5	1.0	0.8
Acetoacetic, mgm	3.0	1.0	1.2	15.0	3.5	19.0
Oxybutyric mgm.	16.3	7.4	Lost	81.5	26.0	231.0

The urine was extracted and examined for optical activity by the method of Black,³³ and found to be very slightly dextrorotatory. The extracts of blood and liver were optically inactive.

Injection experiments on suckling pigs.

EXPERIMENT 5. Two pigs from the same litter were selected. No. 1, weight 5 kgm., and No. 2, weight 4.5 kgm. After three days starvation pig 1 received subcutaneously a solution of sodium acetoacetate equivalent to 4.00 grams of acetone, and pig 2 received an equivalent amount of optically inactive sodium oxybutyrate. Blood samples were taken just preceding the injection and at intervals following it. The results of the analyses³⁴ of the blood appear in Table III.

³¹ Determinations carried out in a similar manner, in which a known amount of acetone was volatilized with the breath, gave quantitative results.

³² Haldane and Smith: *Journ. of Physiol.*, xxv, p. 331, 1900.

³³ *Loc. cit.*

³⁴ 2-cc. samples of blood were taken from an ear vein for each analysis.

TABLE III.

Experiment 5. Injection of sodium acetoacetate and of dl-sodium oxybutyrate into pigs. Analysis of blood samples taken at intervals. Results expressed in terms of milligrams of acetone per 100 grams of blood.

TIME OF TAKING OF BLOOD SAMPLES	FIG NO. 1. SODIUM ACETOACETATE INJECTED = 4.0 GRAMS ACETONE		FIG NO. 2. dl-SODIUM OXYBUTYRATE INJECTED = 4.0 GRAMS ACETONE	
	Acetone + acetoacetic acid	Oxy- butyric acid	Acetone + acetoacetic acid	Oxy- butyric acid
Before injection.....	0.39	0.57	0.34	0.63
2-3 hours after injection.....	4.50	5.20	1.95	18.40
4-5 hours after injection.....	2.80	2.11	0.89	5.20
24 hrs. after injection.....	1.26	2.47	0.28	1.40
48 hrs. after injection.....	1.15	3.90	0.27	2.50

Injection of acetone bodies into phlorhizinized dogs.

Injection of sodium acetoacetate. EXPERIMENT 6. Three female dogs, A, B, and C, weighing respectively 5.4, 6.4, and 7.9 kgm. were used. The animals were put in metabolism cages and catheterized daily. After twenty-four hours fast³⁵ each dog received subcutaneously 1 gram of phlorhizin suspended in olive oil. Two days later, each animal was injected intravenously with a freshly prepared solution of sodium acetoacetate. The solutions were warmed to 37° and run into the jugular vein through a needle, at the rate of about 10 cc. a minute. Each dog received acetoacetic acid equivalent to 6.17 grams of acetone. In the case of dog A, the volume of the solution was 100 cc. and with the others, 200 cc. Within a few minutes the animals had apparently recovered from the shock of the injection. Subsequently large amounts of urine were passed and the animals drank much water for several hours. The results appear in Table IV.

³⁵ Fasting was continued throughout the experiment.

TABLE IV.

Experiment 6. Phlorhizinized dogs injected intravenously with solutions of sodium acetoacetate. Urine analyses. Oxybutyric acid and acetoacetic acid expressed in terms of acetone.

24 HOUR PERIOD ENDING 10 A.M.	URINE VOL- UME cc.	GLUCOSE grams	TOTAL NITRO- GEN grams	D:N	ACETONE AND ACETOACETIC ACID grams	OXYBUTYRIC ACID grams	REMARKS
Dog A							
Oct. 28	240	26.4	6.8	3.8			{ Oct. 29, 3 p.m. injected sodium acetoacetate equivalent to 6.17 gm. acetone.
Oct. 29	200	20.0	5.8	3.4	0.11	0.47	
Oct. 30	800	24.0	7.3	3.3	3.06	1.78	
Dog B							
Nov. 6	253	21.0	6.1	3.4	0.03	0.06	{ Nov. 6, 3:30 p.m. injected acetoacetate equiva- lent to 6.17 gm. acetone.
Nov. 7	1110	16.6	5.5	3.0	3.43	1.95	
Nov. 8	320	13.3	4.9	2.7	0.30	0.90	
Dog C							
Nov. 6	334	27.7	7.3	3.8	0.01	0.03	{ Nov. 6, 4 p.m. injected sodium acetoacetate.. equivalent to 6.17 gm. acetone.
Nov. 7	920	20.8	9.6	2.2	1.87	0.96	
Nov. 8	280	13.3	5.9	2.3	0.08	0.34	

Injection of l-sodium oxybutyrate. EXPERIMENT 7. The same dogs were used as in Experiment 6 and the conditions of the experiment were the same with the exception that 200 cc. of a solution of the sodium salt of l-oxybutyric acid³⁶ were injected into each animal instead of the acetoacetate solutions as in Experiment 6. Each dog received an amount of oxybutyric acid equivalent to 3.54 grams of acetone. The results appear in Table V.

³⁶ Prepared from diabetic urines.

TABLE V.

Experiment 7. Phlorhizinized dogs injected intravenously with solutions of l-sodium oxybutyrate. Urine analysis. Acetoacetic acid and oxybutyric acid expressed in terms of acetone.

24 HOUR PERIOD ENDING 10 A.M.	URINE VOL- UME cc.	GLUCOSE grams	TOTAL NITRO- GEN grams	D:N	ACETONE AND ACETOACETIC ACID grams	OXYBUTYRIC ACID grams	REMARKS
Dog A							
Dec. 4	560	11.8	6.1	1.9	0.43	2.57	{ Dec. 4, 3 p.m. injected intravenously l-sodium oxybutyrate equivalent to 3.54 gm. acetone.
Dec. 5	1150	12.1	5.6	2.2	0.70	5.65	
Dec. 6	400	11.4	5.2	2.2	0.48	1.60	
Dog B							
Dec. 4	410	13.3	4.9	3.1	0.54	2.09	{ Dec. 4, 3:30 p.m. injected intravenously l-sodium oxybutyrate equivalent to 3.54 gm. acetone.
Dec. 5	895	13.2	4.0	3.3	0.66	5.05	
Dec. 6	300	9.1	3.3	2.8	0.55	2.19	
Dog C							
Dec. 4	250	19.4	6.9	2.8	0.09	0.43	{ Dec. 4, 4 p.m. injected intravenously l-sodium oxybutyrate equivalent to 3.54 gm. acetone.
Dec. 5	475	13.0	6.0	2.2	0.17	1.95	
Dec. 6	Urine partly lost.						

Injection of dl-sodium oxybutyrate. EXPERIMENT 8. Same dogs and same conditions as in Experiment 7 except that solutions of dl-sodium oxybutyrate were used. The solutions were the same in strength and quantity as those used in Experiment 7. The results appear in Table VI.

TABLE VI.

Experiment 8. Phlorhizinized dogs injected intravenously with solutions of *dl*-sodium oxybutyrate. Urine analysis. Oxybutyric acid and acetoacetic acid expressed in terms of acetone.

24 HOUR PERIOD ENDING 10 A.M.	URINE VOLUME	GLUCOSE	TOTAL NITROGEN	D:N	ACETONE AND ACETOACETIC ACID	OXYBUTYRIC ACID	REMARKS
	cc.	gms.	gms.		gms.	gms.	
Oct. 16	174	9.9	3.0	3.3	0.14	1.00	{ Oct. 16, 3 p.m. injected intravenously <i>dl</i> -sodium oxybutyrate equivalent to 3.54 gm. acetone.
Oct. 17	570	20.0	5.1	3.9	0.52	4.02	
Oct. 18	366	14.0	5.1	2.7	0.40	2.10	
Oct. 16	306	12.8	4.3	3.0	0.57	2.06	{ Oct. 16, 3:30 p.m. injected intravenously <i>dl</i> -sodium oxybutyrate equivalent to 3.54 gm. acetone.
Oct. 17	664	12.6	4.0	3.1	0.68	4.02	
Oct. 18	240	10.8	3.5	3.1	0.32	1.16	
Oct. 16	312	28.5	8.3	3.4	0.11	0.45	{ Oct. 16, 4 p.m. injected intravenously <i>dl</i> -sodium oxybutyrate equivalent to 3.54 gm. acetone.
Oct. 17	540	25.7	8.4	3.1	0.32	2.08	
Oct. 18	180	12.6	3.5	3.6	0.09	0.27	

Injection of sodium butyrate into normal dogs. EXPERIMENT 9. Puppy weighing 3.4 kgms. was starved for one day and then injected subcutaneously with 25 grams of sodium butyrate dissolved in about 75 cc. of water. Small blood samples were taken at intervals from the external jugular veins and analyzed. The results appear in Table VII.

TABLE VII.

Experiment 9. Injection of 25 grams of sodium butyrate into puppy weighing 3.4 kgm. analysis of blood. Samples taken at intervals. Results expressed in terms of milligrams of acetone per 100 grams of blood.

TIME OF TAKING OF BLOOD SAMPLE	ACETONE AND ACETOACETIC ACID	OXYBUTYRIC ACID
	mgm.	mgm.
Before injection	0.6	0.3
1 hour after injection	5.6	2.2
4 hours after injection	8.0	24.8
6½ hours after injection	1.3	11.8

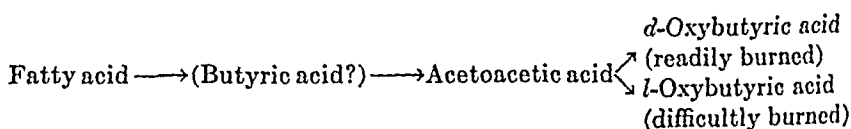
SUMMARY.

The behavior of acetoacetic acid, *l*- and *dl*-oxybutyric acids and butyric acid, in the metabolism, has been studied by means of incubation experiments on hashed organs, and by parenteral introduction into normal and phlorhizinized dogs and young pigs. The analytical results obtained from individual organs, the circulating blood, and the urines have led to the following conclusions:

1. Acetoacetic acid can be readily converted into *dl*-oxybutyric acid in the organism, but the reverse change is difficult, and probably not accomplished under normal conditions.

2. The dextro component of *dl*-oxybutyric acid can be utilized even by the diabetic organism, and this is the explanation of the appearance only of *l*-oxybutyric acid in diabetic urines.

3. The normal path of fatty acid catabolism is as follows:



An hypothesis is presented to explain the mechanism of the burning of oxybutyric acid in the organism.

TURBIDITY METHODS FOR THE DETERMINATION OF ACETONE, ACETOACETIC ACID AND β -OXYBUTYRIC ACID IN URINE.

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(From the Biochemical Laboratories of Harvard Medical School and the Massachusetts General Hospital, Boston.)

(Received for publication, June 1, 1914.)

The application of the principle used in colorimetry to quantitative comparisons of the cloudiness or turbidity produced by suitable colloidal precipitates has long been current in industrial works, and has recently been extended to the determination of (traces of) chlorides (Richards¹), proteins (Kober²), acetone bodies (Marriott³), and fat (Bloor⁴). That turbidity comparisons may be used as a basis for accurate quantitative determinations is of considerable importance, because of the rapidity with which such determinations can be made. In the interest of the development of such analytical technique it would seem desirable not to multiply unnecessarily the optical instruments used. In the so-called nephelometric methods ordinary colorimeters cannot be used because the suspensions are compared on the basis of the amount of light which they reflect and transmitted light must therefore be excluded. We find, however, that perfectly reliable direct turbidity readings can be made with a Dubosecq colorimeter in much the same way as are ordinary color comparisons.

One essential point to be observed in connection with turbidity readings is so to adjust the instrument with reference to the light, that the two fields are perfectly alike when the standard suspension is read against itself. It is less fatiguing to the eye and requires less concentration if the light comes through a hole cut

¹ *Zeitschr. f. anorgan. Chem.*, viii, p. 269, 1895.

² *This Journal*, xiii, p. 485, 1913; *Journ. Am. Chem. Soc.*, xxxv, pp. 290. 1585, 1913.

³ *This Journal* xvi, p. 289, 1913.

⁴ *Ibid.*, xvii, p. 377, 1914.

in an ordinary dark (green) window shade. The numerous more or less bright outdoor objects interfere with the observer's concentration, unless thus excluded, rather more in mere turbidity comparisons than when characteristic colors are measured. But with this simple modification, a modification which is also useful in colorimetric work, turbidities can be matched with as great certainty as colors, provided that the degree of turbidity is regulated to suit the range of the instrument.

A very important special precaution to be observed in connection with turbidity measurements is that a uniform suspension must be obtained without shaking. In the case of most colloidal suspensions shaking or other violent agitation produces more or less coalescence of the suspended particles and grossly erroneous results can thus be obtained. In the case of silver chloride for example it appears to be virtually impossible to secure suspensions suitable for quantitative turbidity comparisons by means of the Duboscq colorimeter because of the great tendency of the silver chloride particles to coalesce. Very gentle rotation or inversion must therefore be employed in the mixing of suspensions for quantitative turbidity comparisons.

The determination of acetone in urine.

In Marriott's methods for the determination of the acetone bodies in blood the essential principle lies in the application of the fact that the Scott-Wilson reagent, an alkaline solution of mercuric cyanide and silver nitrate, gives with acetone extraordinarily insoluble colloidal precipitates which are admirably adapted for turbidity comparisons. By a combination of this precipitation with Folin's air current method for the removal of acetone from urine, we find that the free acetone in urine can be determined in a very short time and with a satisfactory degree of accuracy.

The method is as follows: To about 1 cc. of 10 per cent sulphuric acid in a large test tube add enough urine (0.5 to 5 cc.) to give about 0.5 mgm. of free acetone. Place the test tube and contents in a beaker of water previously heated to 35-40°C. and aspirate the acetone by means of a moderately rapid air current into a second test tube containing 10 cc. of a 2 per cent aqueous solution of sodium bisulphite. The bisulphite, we find, holds the

acetone almost as effectively as acids hold ammonia. It has two drawbacks; first, that its solutions do not keep very long (about a week), and secondly that it diminishes the amount of turbidity obtained, so that the same amount of the bisulphite solution must be added to the standard acetone solution in order to secure strictly comparable turbidities. These difficulties are, however, not serious. The sulphite solution can be made in a few minutes as its exact concentration is unimportant, and the turbidity obtained from 0.5 mgm. of acetone after dilution to 100 cc. is ample for accurate readings in the colorimeter.

It is possible to collect the acetone directly in 60-70 cc. of cold water in the 100-cc. volumetric flask, but the air current used for driving over the acetone must then be slower and more carefully regulated, and we prefer therefore to collect the acetone by the help of the sodium bisulphite solution as described.

The aspiration process itself is so simple as scarcely to need description. The arrangement and connection of the test tubes is substantially the same as has been described in connection with the determination of ammonia and nitrogen in urine.⁵

The length of time required for removing all the acetone depends of course on the rapidity of the air current and on the volume of urine used. In general we have found that ten minutes suffices for the removal of 2 mgm. of acetone from 5 cc. of liquid, and it is seldom that as much as 5 cc. of "acetone urine" is required to yield 0.5 mgm of acetone. As soon as the aspiration is completed the bisulphite solution containing the acetone is transferred to a 100-cc. volumetric flask diluted to a volume of 50-60 cc., and to it is then added 15 cc. of the Scott-Wilson reagent. The contents are then diluted to 100 cc. with water and mixed.

The Scott-Wilson reagent is prepared as follows: To 10 grams of mercuric cyanide dissolved in 600 cc. of water add a solution of 180 grams of sodium hydroxide in 600 cc. of water. If the mixture is warm cool it and then add very slowly with constant shaking a solution of 2.9 grams of silver nitrate dissolved in 400 cc. of water. The solution should be set aside for at least three or four days before being used. At the end of this time the small amount of sediment present will have settled, leaving a clear supernatant liquid which may then be siphoned off. No filtration is necessary.

⁵ This *Journal*, xi, p. 499, 1912.

The same amount of the Scott-Wilson reagent (15 cc.) is added to 0.5 mgm. of acetone in the presence of 10 cc. of the bisulphite solution and water enough to make a volume of 50-60 cc. As with the unknown the solution is then at once diluted to 100 cc. and mixed by shaking. The addition of the reagent and the water and the final mixing of the two solutions should be done fairly rapidly, *i.e.*, so that the interval between the preparation of the two is as short as possible. The two flasks are allowed to stand for from twelve to fifteen minutes and the relative turbidities are then determined by means of the colorimeter, care being taken invariably to first read the standard against itself.

Preparation of standard acetone solutions.

In turbidity measurements, as in colorimetric comparisons, one fundamentally important feature is the question of the standard. In the preparation of standard acetone solutions we have found, as was previously noted by Marriott, that dilute acetone solutions when treated with the Scott-Wilson reagent give much more turbidity in the case of freshly distilled solutions than in the case of undistilled acetone. In connection with his acetone determinations in blood Marriott was therefore compelled to employ as standards only freshly distilled solutions. Even dilute acetone solutions seem to polymerize very rapidly to a stage where they do not give quantitative precipitates with the Scott-Wilson reagent. After considerable experimentation we have found that freshly distilled acetone diluted with $\frac{N}{4}$ sulphuric acid (until 10 cc. contains only 0.5 mgm. of acetone) keeps for several weeks, but we are not yet prepared to say that such solutions keep indefinitely. In more concentrated solutions of acetone $\frac{N}{4}$ sulphuric acid retards but does not prevent the polymerization.

The preparation of the standard acetone solution is accordingly made as follows:

About 2 cc. of Kahlbaum's purest acetone (from the bisulphite compound) is diluted with about 500 cc. of water and distilled. About 150 cc. of the distillate is collected in a large flask containing about 100 cc. of $\frac{N}{4}$ sulphuric acid. The distillate is diluted to about a liter with $\frac{N}{4}$ sulphuric acid and its exact acetone content is then determined in the usual way by titration with iodine

and thiosulphate. On the basis of this titration a part of the solution is at once diluted with $\frac{N}{4}$ sulphuric acid so that 10 cc. contains exactly 0.5 mgm. of acetone.

For subsequent preparations of the dilute standard solution from the titrated stock solution the required amount of the latter is measured into a flask, distilled and diluted with sulphuric acid and water until it contains 0.5 mgm. of acetone in 10 cc. and is an approximately fourth-normal solution of sulphuric acid.

The determination of acetoacetic acid.

Acetone urines contain from two or three to nine or ten times as much acetoacetic acid as acetone. In strictly fresh urines the latter proportions prevail, but the older the urine the greater becomes the relative proportion of acetone because of the spontaneous decomposition of the acetoacetic acid. Because of the relatively small amounts of acetone we find it more convenient to determine the sum of the acetone and acetoacetic acid in a separate sample of urine than to use the sample measured out for the determination of the preformed acetone. Urines giving a strong ferric chloride reaction usually contain more than 0.5 mgm. of acetoacetic acid per cubic centimeter and must be diluted so that an appropriate fraction of 1 cc. (of the original urine) can be taken for a determination. The amount of urine taken should yield approximately 0.5 mgm. of acetone (from 0.3 to 0.7 mgm.). This amount of urine is transferred to a large test tube containing 1 cc. of 10 per cent sulphuric acid and is connected in the usual way with a second test tube containing 10 cc. of the 2 per cent sodium bisulphite solution. The test tube containing the urine is then immersed in a beaker of boiling water while an *extremely* slow air current is passing through. At the end of ten minutes the speed of the air current should be increased, but need not be made very rapid. The heating and aspiration is then continued for another five minutes when the acetoacetic acid plus acetone is all found in the form of acetone in the bisulphite solution. The latter is then transferred to a 100-cc. volumetric flask and the acetone is determined exactly as in the determination of the preformed acetone.

One milligram of acetone is equivalent to 1.8 mgm. of aceto-

acetic acid. From the total acetone of the 24-hour quantity of urine is subtracted the total preformed acetone, and the remainder multiplied by 1.8 gives the acetoacetic acid.

The determination of β -oxybutyric acid.

The method which we have found satisfactory for the determination of β -oxybutyric acid in urine, like the method described by Marriott for blood, consists of an adaptation of Shaffer's well known oxidation method with turbidity determinations of the acetone formed. According to Shaffer's most recent paper⁶ on the oxidation of β -oxybutyric acid with chromic acid there is a loss of from 5 to 10 per cent in the yield of acetone obtained. Ten per cent is a large correction for use in connection with a quantitative determination and we hoped that in this case, as in many others, a more nearly quantitative transformation might be obtained by working with very small amounts of substance. Marriott evidently made no investigations of the oxidations of oxybutyric acid from this point of view, and accordingly makes use of the same 10 per cent correction in connection with his determinations of β -oxybutyric acid in blood as he and Shaffer used in determining larger amounts, 50–60 mgm. of the substance.⁷ As good luck would have it oxidation of 1–2 mgm. of β -oxybutyric acid with boiling chromic acid solutions yields the theoretical amounts of acetone. We have made a large number of such oxidation experiments with a repeatedly purified calcium zinc salt of β -oxybutyric acid obtained from Shaffer, and have found that it is not even necessary to introduce the bichromate gradually as in Shaffer's original method in order to get the theoretical yield of acetone. On the basis of this finding the determination of β -oxybutyric acid becomes almost as simple and convenient as the determination of acetone or of acetoacetic acid.

As actually carried out with urine the determination is a little different according to whether the urine does or does not contain sugar in addition to the acetone bodies. For ordinary diabetic urines containing sugar the determination is as follows:

Since most acetone urines contain several milligrams of β -oxy-

⁶ This *Journal*, xvi, p. 265, 1913.

⁷ This *Journal*, xvi, p. 298, 1913.

butyric acid per cubic centimeter and since not more than 2 mgm. are wanted for the determination, a portion of the urine must first be diluted to from 10 to 50 times its original concentration. The degree of dilution required can be learned only by experience but the qualitative tests for acetone and acetoacetic acid are valuable as an index. Diluted urine, equivalent to 2-4 mgm. of oxybutyric acid, is measured into a 500-cc. Kjeldahl flask. About 200 cc. of water and 5 cc. of 10 per cent sulphuric acid are added and the mixture is boiled for a few (ten) minutes to remove the preformed acetone and the acetoacetic acid. To the contents in the flask is then added (with a cylinder) 25 cc. of a solution containing 2 per cent potassium bichromate and 35 per cent sulphuric acid; the flask is immediately connected with the condenser and the mixture is distilled slowly for from forty to sixty minutes.⁸ Since acetone is very volatile and its formation is very slow nothing is gained by making the distillation rapid, in fact it is best to bring the contents of the distilling flasks to the boiling point as rapidly as possible and then to turn down the flame to such a point that practically no distillate is obtained. After continuing this slow heating for about thirty minutes the flame is again increased and the liquid rapidly distilled for about fifteen minutes. No more than 80-125 cc. of distillate should be obtained in the time indicated. The distillate is collected in another 500-cc. Kjeldahl flask containing about 75-100 cc. of cold water. During the entire distillation the tip of the delivery tube should be kept below the surface of the liquid in the receiver. If this detail be overlooked loss of acetone will frequently take place. To the distillate is added a couple of grams of sodium peroxide and the distillation is then repeated and continued until about 80 cc. of distillate is obtained. This distillate is collected in a 100-cc. measuring cylinder containing about 10 cc. of water. This second distillation can be finished in from ten to fifteen minutes.

The distillate is then diluted to 100 cc., mixed, and from 25 to 50 cc. is measured into a 100-cc. volumetric flask containing about 25 cc. of water. The Scott-Wilson reagent (15 cc.) is then added and the mixture is diluted with water to the mark and mixed. To another 100-cc. flask containing 0.5 mgm. of acetone

⁸ Any of the ordinary makes of Kjeldahl stills may be conveniently used for this distillation provided that good condensation is secured.

and about 50 cc. of water is similarly added 15 cc. of Scott-Wilson reagent and water. The reaction with the standard should be made as soon as possible before or after the unknown distillate has been mixed with the precipitant. The two flasks are then allowed to stand for from ten to fifteen minutes and the turbidities are compared as in the determination of the preformed acetone. It will be noted that in this case no sodium bisulphite is used. Each milligram of acetone corresponds to 1.78 mgm. of β -oxybutyric acid.

In Shaffer's method the glucuronic acid and nearly all the sugar are precipitated by means of a preliminary treatment with basic lead acetate and ammonia, because it was found (Shaffer and Emden and Schmitz) that these substances on oxidation with chromic acid yield products which pass into the final distillate and which take up iodine during the titration of the acetone. According to our results all the oxidation products obtained from sugar and which react with the Scott-Wilson acetone reagent are destroyed or removed by the second distillation with the alkaline peroxide mixture, and the rather tedious precipitation with basic lead acetate has therefore been omitted.

In the case of urines containing no sugar such as are frequently obtained from children and from fasting persons the determination of the β -oxybutyric acid does not require a second distillation. The first distillation is made in the same way as when sugar is present, except that it must be so slow as not to yield more than about 80 cc. of distillate in from forty to sixty minutes. This distillate is collected directly in a 100-cc. measuring cylinder (containing about 10 cc. of water), and is at once diluted to 100 cc. and mixed. To it is then added 1-2 grams of sodium peroxide, and after further mixing by means of a few inversions the solution is ready for the precipitation of the acetone. Repeated determinations of known amounts of oxybutyric acid, added to normal urine, in the form of the zinc calcium salt, have shown that the disturbing effects of the normal urinary constituents are removed by the treatment with sodium peroxide without a second distillation. When sugar is present the second distillation cannot be avoided as the results obtained are otherwise much too high.

In the table below are given a series of analytical results obtained from urine containing added known quantities of dextrose and β -oxybutyric acid.

Sugar and β -oxybutyric acid in grams per liter of urine.

DEXTROSE ADDED	β -OXYBUTYRIC ACID ADDED	β -OXYBUTYRIC ACID RECOVERED	
		Grams	Per cent
100	17.76	17.40	98.0
100	17.76	17.33	97.6
50	17.76	17.76	100.0
50	17.76	17.61	99.2
50	35.52	35.52	100.0
50	35.52	35.52	100.0
25	8.88	8.79	99.0
25	8.88	8.83	99.5
25	8.88	8.88	100.0
25	8.88	8.88	100.0
25	17.76	17.93	101.0
25	17.76	17.58	99.0
5	17.76	17.23	97.0
5	17.76	17.76	100.0
0	17.76	17.76	100.0
0	8.88	8.79	99.9
0	3.50	3.53	101.0

THE QUANTITATIVE DETERMINATION OF ALBUMIN IN URINE.

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No abnormal urinary constituent is so frequently tested for qualitatively as albumin, yet there is curiously enough no reasonably accurate and convenient method available for its quantitative determination. The clinical Esbach method is rapid, but, like all "sedimentation" methods, is wholly untrustworthy. And the coagulation methods with accompanying filtrations, washings and weighings, or nitrogen determinations, are so tedious and laborious that they are very seldom used. Whether or no quantitative determinations of albumin in urine are capable of yielding much valuable information suitable methods for the determination of this abnormal urinary constituent are manifestly needed. In this paper we shall describe two fairly convenient methods neither of which is particularly original but both of which appear to give satisfactory results.

Turbidity method. Kober¹ has described a nephelometric method for the determination of proteins in milk and in digestion mixtures, but so far as we know the principle has not yet been applied to the determination of albumin in urine.

As applied to urine the turbidity method for the determination of albumin is as follows:

To about 75 cc. of water in each of two 100-cc. volumetric flasks is added 5 cc. of a 25 per cent solution of sulphosalicylic acid.² To one flask is then added 5 cc. of the standard protein

¹ This *Journal*, xiii, p. 485, 1913; *Journ. Am. Chem. Soc.*, xxxv, p. 290, 1913.

² After having tried out a number of different precipitants for albumin, we came to the conclusion previously reached by Kober, namely that sulphosalicylic acid is the best reagent for the quantitative precipitation of albumin.

solution containing 10 mgms. of albumin, and to the other is added the albuminous urine 1 cc. at a time (by means of an Ostwald pipette) until the turbidity obtained seems to be reasonably near that of the standard. The two flasks are then filled up to the mark with water, cautiously inverted a few times to secure mixing, and are then ready for the quantitative comparison in the colorimeter tubes. The standard must invariably first be read against itself to secure the adjustment of the colorimeter (and of the eye). The contents of one of the Duboseq colorimeter cups is then replaced by the suspension of the unknown, and the turbidity comparison is made exactly as in colorimetric work.

The standard containing 10 mgms. of protein is set at 20 mm. The unknown must not read less than 10 nor more than 30 mm.

Dividing 200 by the product of the reading of the unknown and the number of cubic centimeters of urine taken gives the albumin in milligrams per cubic centimeter of urine. It is very important not to shake the albuminous suspensions in the volumetric flasks because of the tendency of the precipitate to agglutinate. The preliminary mixing must therefore be accomplished by means of a few gentle inversions.

A quantitative albumin determination can be made with a very satisfactory degree of accuracy in the course of a few minutes in the manner described provided only that a standard albumin solution is available.

The standard protein solution is prepared from fresh blood serum free from haemoglobin. For the preparation of this serum either slaughter house or normal human blood may be used. The so-called blood serum sold for the preparation of bacteriological culture media should be avoided as it is usually several days old and is frequently partially decomposed. The dried preparations of "blood albumin" listed by chemical dealers are also not satisfactory for the preparation of standard solutions.

To prepare the standard 25-35 cc. of serum are diluted with a 15 per cent solution of chemically pure sodium chloride to about 1500 cc. The solution is mixed and filtered. By means of nitrogen determinations the protein content of the filtrate is determined ($\text{protein} = N \times 6.25$) and on the basis of the figure obtained the solution is diluted with 15 per cent sodium chloride solution so that it contains 2 mgms. of protein per cubic centimeter.

Sodium chloride in the concentration mentioned is fairly effective as a preservative. Nevertheless it is best to saturate the standard albumin solution with chloroform (20 cc.). We have had several different protein standards in the laboratory for some months and have been unable to find any change. As a matter of further precaution we have made it a practice, however, to keep the stock solutions in a refrigerator.

The above method is not applicable to urines which are very deeply colored with blood or bile pigments. The method is of course applicable to other albuminous fluids than urine as for example exudates, transudates and the cerebrospinal fluid.

Gravimetric method. The gravimetric determination of albumin in urine here described was devised for the purpose of checking up the values obtained by the preceding turbidity method and differs only in minor details from the gravimetric determinations long since described by others. It is as follows:

Ten cubic centimeters of urine are pipetted into an ordinary conical centrifuge tube which has been previously weighed. To this urine is then added 1 cc. of 5 per cent acetic acid and the tube allowed to stand for fifteen minutes in a beaker of boiling water. At the end of this time the tube is removed from the water bath and centrifuged for a few minutes. The supernatant liquid is then poured off, the precipitate in the tube is stirred up with about 10 cc. of boiling 0.5 per cent acetic acid and again centrifuged. The supernatant liquid is then again poured off and the precipitate in the tube again washed, this time with 50 per cent alcohol. After centrifuging and pouring off the supernatant liquid, for a third time the tube is placed for two hours in an air bath at 100–110°, then cooled in a desiccator and weighed.

The following results were obtained by means of the gravimetric method on sodium chloride solutions to which standardized solutions of blood serum had been added:

Grams per liter of protein added.	Grams per liter of protein found.
1.30	1.22
1.05	1.03
1.05	1.00
1.05	1.02

The following results were obtained on a number of albuminous urines taken at random from material sent in to the hospital laboratory.

URINE NO.	GRAMS PROTEIN PER LITER BY THE GRAVIMETRIC METHOD	GRAMS PROTEIN PER LITER BY THE TURBIDITY METHOD
1	3.8	4.4
2	1.5	1.6
3	1.2	0.9
4	3.4	3.2
5	3.4	3.5
6	5.3	4.8
7	4.1	4.5
8	3.8	3.5
9	7.1	6.8
10	5.2	5.4
11	4.4	4.7
12	3.6	3.5
13	4.2	4.5
14	2.3	2.5
15	0.95	0.92
16	0.8	0.80
17	0.40	0.42
18	2.0	2.1

METABOLISM IN BENCE-JONES PROTEINURIA.

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Although the object of study for more than sixty years considerable theoretical interest is still attached to the condition known as Bence-Jones proteinuria, not only because of its comparative rarity but on account of the fact that our knowledge of the origin of the body known as Bence-Jones protein is still very incomplete. On account of the small number of cases on which any systematic observations have been made every example of this interesting condition may still be looked upon as offering a field for investigation.

To account for the origin of the Bence-Jones protein three theories have been advanced. According to the earliest of these this peculiar protein is manufactured in the diseased areas of bone marrow, and being a "foreign protein" is on passage into the blood stream excreted unchanged by the kidneys. The second theory was that proposed in 1900 by Magnus-Levy.¹ According to this investigator it is inconceivable that the relatively small areas of diseased bone marrow should be able to produce the large amounts of Bence-Jones protein found in the urine in some cases. He therefore suggests that this protein is formed in the intestine as the result of abnormal and unknown conditions present in the bowel. If this theory be true there should be a direct relation between the amount and possibly the kind of protein in the diet and the amount of excreted albumose, a relation which Magnus-Levy believed he was able to demonstrate.

As it happens "Witte's Peptone" and other peptic digestion mixtures do contain large quantities of an albumose,² which in

¹ *Zeitschr. f. physiol. Chem.*, xxx, p. 237, 1900.

² Folin: *ibid.*, xxv, p. 154, 1898.

its solubilities and reactions corresponds very closely to the solubilities and reactions of the Bence-Jones protein, a fact, which however, was overlooked by Magnus-Levy as well as by all subsequent investigators. The hypothesis that the Bence-Jones albumose comes directly from the digestive tract has not received support by later investigations, and it seems less plausible now than it might have seemed when first advanced, because of the changed views concerning the character of protein digestion and absorption which now prevail. The product might still be explained as a sort of digestion product, but notwithstanding Magnus-Levy's calculations the digestion involved must then be a pathological autolytic internal digestion, which has nothing to do with the specialized normal digestive processes of the bowel.

The third hypothesis advanced in 1900 by F. Voit and Salvendi,³ is in some respects the reverse of the hypothesis formulated by Magnus-Levy, for according to this hypothesis the eliminated albumose comes from the body protein and not from the food protein. Abundant food, particularly nitrogenous food which would "spare" body protein, according to this view would bring about a reduction in the formation and elimination of the pathological metabolism product. This point of view has remained a pure speculation, for neither its authors nor anyone else have used it as a working hypothesis in metabolism studies of the disease.

The relation of the protein intake to the output of Bence-Jones protein was first studied by Allard and Weber.⁴ More recently Hopkins and Savory⁵ in the course of a most comprehensive and illuminating investigation on the character, composition and metabolic origin of Bence-Jones albumose came to the conclusion, previously reached by Allard and Weber, that the amount of protein taken with the food has no direct influence on the amount of excreted albumose, though they incline to the view that the general level of the metabolism does have more or less indirect influence on the amounts of excreted protein.

We have recently had under observation in the medical wards of the Massachusetts General Hospital a case of multiple myeloma of the ribs, and have been able to make under carefully controlled

³ *Münch. med. Wochenschr.*, 1904, p. 1281.

⁴ *Deutsch. med. Wochenschr.*, 1906, p. 1251.

⁵ *Journ. of Physiol.*, xlii, p. 189, 1911.

conditions a few observations of the effect of diet on the excretion of the Bence-Jones protein.

The patient M. G., a Russian Jew, 39 years old, by trade a tailor, states that about eleven months before his entry into the hospital he began to be troubled by severe intermittent pain in the precordia. This pain has gradually spread to various parts of the chest and at the time he came under our observation had become so severe that he was obliged to remain in bed as the slightest movement caused the most intense pain.

Physical examination showed a well developed and well nourished man weighing 140 pounds, who showed several tender spots in both lower axillae along the course of the 7th, 8th, 9th, and 10th ribs. X-ray examination showed marked lesions of the bone in a number of areas on the ribs. The heart and lungs were normal. The Wasserman test of the blood was negative, and there was no evidence of tuberculosis or nephritis. Examination of the urine showed a few hyaline and granular casts and a large amount of protein which gave the reactions typical of the Bence-Jones albumose. On warming the protein coagulated between 40° and 55°C. and entirely redissolved on boiling. Addition of mineral acids produced a precipitate, which redissolved on boiling and which reappeared on cooling if too large an excess of acid had not been used. With potassium ferrocyanide and acetic acid no precipitate was obtained except after very long standing. Examination of the urine on a number of days has shown the protein to be invariably present, and to consist entirely of the Bence-Jones albumose.

Our determinations of the Bence-Jones albumose were made by the following method. Ten cubic centimeters of urine were placed in a conical previously weighed centrifuge tube, 1 cc. of 5 per cent acetic acid was added, and the tube left overnight⁶ in a water bath at 60°C. The next morning the tubes were removed from the bath, centrifuged for a few minutes and the supernatant liquid poured off. The sediment was then well stirred up with about 10 cc. of 50 per cent alcohol, the tubes again centrifuged, and after pouring off the supernatant liquid were dried at 100°C., cooled and weighed. The above method is a modification of the method used by Hopkins and Savory in their albumose determinations.

Our object was primarily to study the effects of different levels of protein intake on the albumose excretion, but in order to make the metabolism record more complete we have included some more detailed analyses of the urine.

⁶ The determinations were usually started at almost six o'clock in the afternoon so that the tubes could be removed from the bath at about eight o'clock the following morning,

During the first period the patient was placed for six days on a diet low in protein and of high calorific value. The daily ration contained 300 grams of arrowroot starch, 500 cc. of 30 per cent cream, 50 grams of butter, 50 grams of cane sugar, 100 grams of white bread, from 300 to 400 grams of baked potato or boiled rice, one raw apple and one orange. The fuel value of this diet as calculated from Atwater's tables amounted to an average of 3000 to 3500 calories per day. The results of the analyses of the urine during this period are given in Table I.

TABLE I.
Urinary nitrogen.

DATE, JAN.....	19-20	20-21	21-22	22-23	23-24	24-25
Volume, cc.....	2040	1370	1700	1960	1540	1630
Sp. gr.....	1.0120	1.0125	1.0110	1.0105	1.0110	1.0110
Bence-Jones protein, gm.....	2.61	2.66	2.80	1.76	1.96	2.77
Total N, gm.....	8.36	6.61	6.10	5.70	5.51	5.00
Total non-protein N, gm.....	7.95	6.19	5.66	5.44	5.19	4.56
Protein N, gm.....	0.41	0.42	0.44	0.26	0.31	0.44
Urea N, gm.....	6.29	4.97	4.00	3.77	3.76	3.40
Ammonia N, gm.....	0.43	0.36	0.61	0.35	0.35	0.21
Creatinine N, gm.....	0.45	0.39	0.37	0.37	0.39	0.35
Creatine N, gm.....	0	0	0	0	0	0
Uric acid N, gm.....	0.16	0.13	0.19	0.15	0.13	0.13
Rest N, gm.....	0.62	0.34	0.51	0.63	0.56	0.47

Per cent of total nitrogen.

Protein.....	4.8	6.3	7.2	4.5	5.6	8.8
Urea.....	79.1	75.3	65.5	69.4	68.2	68.0
Ammonia.....	5.2	5.8	10.8	6.4	6.7	4.6
Creatinine.....	5.3	5.9	6.0	6.4	7.0	7.0
Creatine.....	0	0	0	0	0	0
Uric acid.....	1.8	1.9	3.1	2.6	2.3	2.6
Rest.....	7.4	5.1	8.3	10.1	10.1	9.4

The period of low protein feeding was followed by one in which a purine-free, but extremely liberal diet as high in protein as the patient could be induced to take was given. During this period the protein was supplied largely in the form of eggs, milk and

cheese. The results obtained on analysis of the urine during this period are given in Table II.

The notably increased elimination of the Bence-Jones albumose during this period of high protein feeding indicated that the formation of the pathological metabolism product is indeed greatly affected by the protein contents of the diet. During the first

TABLE II.

DATE, JAN.....	25-26	26-27	27-28	28-29	29-30	30-31	31- FEB. 1	FEB. 1-2
Volume, cc.....	1190	1310	1600	1150	1050	1300	1140	1570
Sp. gr.....	1.018	1.021	1.016	1.022	1.025	1.025	1.029	1.025
Bence-Jones protein, gm.....	3.70	5.45	4.39	6.33	5.54	6.10	6.04	5.00
Total N, gm.....	6.57	8.72	9.40	11.50	13.10	15.62	15.71	16.60
Total non-protein N, gm.....	5.92	7.85	8.70	10.49	12.22	14.65	14.75	15.80
Protein N, gm.....	0.59	0.87	0.70	1.01	0.88	0.97	0.96	0.80
Urea N, gm.....	3.81	6.17	6.85	7.68	10.17	10.59	11.76	12.80
Ammonia N, gm.....	0.79	0.43	0.57	1.03	0.73	0.72	0.78	0.86
Creatinine N, gm....	0.33	0.33	0.28	0.37	0.35	0.29	0.31	0.39
Creatine N, gm.....	0.04	0.08	0.12	0.05	0.04	0.14	0.08	0
Uric acid N, gm.....	0.14	0.13	0.15	0.14	0.13	0.14	0.15	0.16
Rest N, gm.....	0.80	0.62	0.73	1.22	0.80	1.75	1.66	1.60

Per cent of total nitrogen.

Protein.....	9.0	10.0	9.3	8.8	6.7	6.2	6.1	4.8
Urea.....	58.6	70.9	73.0	66.7	77.6	74.1	74.9	77.1
Ammonia.....	13.3	5.5	6.8	9.9	5.9	4.9	5.3	5.1
Creatinine.....	5.0	3.7	2.8	3.2	2.6	1.8	1.9	2.3
Creatine.....	0.6	0.9	1.2	0.4	0.3	1.0	0.5	0
Uric acid.....	2.1	1.4	1.5	1.2	1.0	0.9	0.9	0.9
Rest.....	12.1	7.1	7.6	10.4	6.1	11.2	10.5	9.6

period we found from 1.76 to 2.8 grams of albumose in urines containing from 5 to 8.5 grams of total nitrogen, while in the second period the albumose elimination rose to 6.5 grams and the total nitrogen to 16 grams. Considered more in detail it is, however, equally clear that there is nothing like a definite or constant relationship between the total nitrogen and the albumose in the urine. The increased albumose elimination during the period of high protein feeding might therefore have been a mere coincidence.

The patient expressed himself as feeling unusually well during the period covered by the low nitrogen feeding experiments, but he had a relapse and felt much worse during the following feeding experiments with a liberal diet. An interesting fact to be noted in this connection is the considerable creatine elimination during the period of high protein feeding.

Hopkins and Savory also found creatine in the urine of their patients and seem to consider the creatine elimination a constant phenomenon in multiple myeloma. Creatine has now been found in so many different conditions that its occurrence in this disease is scarcely any more than one should expect. Its occurrence is, however, by no means constant. We found none of it during the first metabolism period, and we are inclined to ascribe the occurrence and the amount of the creatine appearing in the urine as something of an index to the subjective condition of the patient. Because of the condition of the patient we were unfortunately not able to carry out our plan to return to the original low nitrogen diet of the first period. He could not take it, and in fact could take very little food of any kind.

During the three days immediately following the high protein feeding we found 6, 7.9 and 4.4 grams respectively of albumose in the urine. The total nitrogen of these days amounted to 13.4, 13.4 and 8.6 grams. In view of these facts we must conclude, as did Hopkins and Savory, that the protein content of the food has no direct bearing or influence on the elimination of the Bence-Jones albumose.

In view of the patient's poor appetite we felt justified in requesting him to abstain from taking any food for a couple of days. He did so and we obtained 5.2 and 5 grams respectively of albumose and 8.8 and 8.4 grams of (non-protein) nitrogen in the urine. In considering the results obtained during this period of starvation it occurred to us that the effect produced on the albumose excretion by the ingestion of food might be studied by examining separately the day and night urines. For a few days the urines were therefore collected in twelve hour periods from 7 a.m. to 7 p.m. and from 7 p.m. to 7 a.m. The last meal of the day, a light one, was given at 5 p.m., and the patient received no food until 8 o'clock the next morning. As will be seen by the figures given in Table III the excretion of protein is practically the same during the two periods.

TABLE IV.

DAY	TIME	VOLUME	TOTAL NITROGEN	BENCE-JONES PROTEIN
			grams	grams
1	Day.....	590	9.7	2.9
	Night.....	260		2.3
2	Day.....	350	9.2	2.5
	Night.....	360		2.5
3	Day.....	360	9.9	2.6
	Night.....	300		1.7
4	Day.....	315	10.3	2.0
	Night.....	370		3.1
5	Day.....	330	8.0	2.0
	Night.....	320		2.7
6	Day.....	340	6.8	2.3
	Night.....	320		2.7

To account for the albumose elimination during these two days of complete starvation following a period of inadequate feeding on the basis of the hypothesis of Magnus-Levy would be rather difficult, unless one is willing to fall back on the dramatic but improbable hypothesis of Freund that in fasting the protein is secreted into the intestine and there digested before being reabsorbed (in the form of amino-acids).

Aside from the creatine elimination when the subjective symptoms become severe the protein metabolism in multiple myeloma appears to be normal so far as the ordinary normal nitrogenous metabolism products are concerned.

The Bence-Jones albumose is more or less similar to one of the primary peptic digestion products (Meissner's "metapeptone") and is probably formed by internal autolytic digestion since it appears to be independent of the total protein metabolism. From this point of view it would seem not altogether improbable that in some cases of multiple myeloma traces of other albumoses might occur in the urine, but in our case, as in the two cases investigated by Hopkins, no such albumoses were found. Like Hopkins and Savory⁷ we have been unable as yet to obtain our Bence-Jones albumose in crystalline condition by means of the method of Gritterink and de Graaff.⁸

⁷ *Loc. cit.*

⁸ *Zeitschr. f. physiol. Chem.*, xxxiv, p. 393, 1912.

THE IMPORTANCE OF THE LIVER IN UREA FORMATION FROM AMINO-ACIDS.

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The chief methods of attack upon the problem of the site of urea formation have been: (1) comparative determinations of the urea content of blood from various sources and of tissues, and (2) urine analysis (*a*) after operative procedures which exclude the liver more or less completely from the circulation and (*b*) in hepatic disease in man.

Comparative analyses have yielded no conclusive result, for several reasons. The methods used for the various kinds of material are not known to be comparable, the discrepancies lying chiefly in the completeness of extraction. In addition to, and doubtless largely because of this, the results have never been uniform. Finally, there is no obvious connection between site of formation and site of storage.

The question of the amino nitrogen content of the urine in diseases of the liver, especially after the administration of amino-acids, holds at the present day an important position in relation to the functional diagnosis of liver lesions. Here likewise results have varied greatly. Even assuming an excessive excretion of amino nitrogen and a subnormal "assimilation limit" for amino-acids in liver disease, however, there is no proof from this alone that the liver is at all concerned in urea formation.¹

The only operative procedures to be considered in this connection are those which exclude the liver completely from the circulation. Operations of this sort are entirely incompatible with prolonged life. Conclusions drawn from urinary changes under such cir-

¹ This subject has been recently discussed by Fiske and Karsner: this *Journal*, xvi, p. 399, 1913.

cumstances form to a large extent the basis for present notions as to the site of urea formation. The fact has not been sufficiently taken into account that the liver, after such treatment, is not the only organ affected. Renal function, for example, is markedly disturbed (oliguria, albuminuria, hemoglobinuria). We shall show later that demonstrable urea formation from amino-acids, when the blood supply to the liver is ligated, takes place only during a comparatively short period after the operation.

Omitting consideration of arginine, the only well-established facts concerning urea formation from protein cleavage products are: (1) that the liver is not the sole site of its formation, and (2) that the liver does form urea from the ammonia of the portal blood, and is, at least under normal conditions, the chief site of this particular process. In the light of the demonstration by Folin and Denis² that the ammonia of the portal blood is chiefly a bacterial product, its conversion into urea is to be considered merely as one of the numerous protective devices of the organism. It is important to note that, whereas calculations based upon the earlier high values for ammonia in blood could account for the total 24-hour urea excretion (Nencki, Pawlow and Zaleski³), the newer, much lower and undoubtedly more nearly correct analytical figures of Folin and Denis⁴ show clearly that only a small fraction of the urea can be accounted for in this way.

Apart from the above-mentioned facts, nothing definite can be said. Many of the writers on this subject have taken it for granted that urea formation from amino-acids, in its final steps, is identical with and must therefore occur at the same site as that from ammonia. This assumption has no firm foundation on fact. It can not be stated that ammonia is an obligatory intermediary product in the cleavage of amino-acids in the mammalian organism.⁵

The chief purpose of this paper is to present what we believe to be the first demonstration of urea formation from amino-acids in living animals so treated that the participation of the liver is excluded. The main difficulty encountered was that of obtaining

² This *Journal*, xi, p. 161, 1912.

³ *Arch. f. exp. Path. u. Pharm.*, xxxvii, p. 26, 1895.

⁴ *Loc. cit.*

⁵ See Dakin: this *Journal*, vi, p. 235, 1909.

satisfactory controls. Long experiments (three hours) fail of their purpose because of the shock involved. After this length of time it is frequently almost impossible to draw 5 cc. of blood from the carotid artery. On the other hand, sufficient time must be allowed for definite urea formation to take place, and, furthermore, the amino-acid solution must be injected slowly in order to avoid toxic symptoms. The procedure finally adopted was to inject the amino-acid intravenously over a period of fifty minutes, and to take the final samples of blood and tissue ten minutes later. Under these circumstances, the accumulation of urea in the muscle is not great even when the liver is in circulation, but it is equally marked when the liver is excluded. The urea content of the blood rises definitely in both cases, and to about the same extent. In other words, direct experiment, eliminating the confusing effect of variations in renal activity, fails to show that the presence of the liver (or other abdominal organ) in the circulation is essential to the formation of urea from amino-acids. That this process occurs to some extent in the liver, however, we are in no position to doubt.

The claim that the liver represents the chief site of urea formation has always been open to question. All the evidence from which this claim has derived support is subject to objection upon the ground that the experiments have been only apparently controlled.⁶ The data herein presented, leading to the opposite result, are comparatively free from this objection. We realize, of course, that a substance injected into an animal which is deprived of the circulation through its abdominal viscera is distributed over a smaller mass of tissue than the same amount injected into a normal animal, and hence undergoes less dilution. On the other hand, if, as appears likely, urea formation is a general function of mammalian protoplasm, the substance in the first case is brought into contact with less tissue which is capable of acting upon it. It is unfortunate that the conditions prohibit longer experiments and mathematically accurate controls, but the procedure we have adopted is obviously a step in advance of urine analysis for this purpose.

⁶ This includes urine analyses in liver disease. Normal subjects are not proper controls in such investigations, and yet, so far as we are aware, they are universally used for that purpose.

EXPERIMENTAL PART.

Cats were anesthetized with ether, between eighteen and twenty-four hours after their last meal (fish), a tracheal cannula immediately inserted and artificial respiration instituted. The details of the experiments varied slightly, but in no essential points except as described. Samples of blood (5 cc.), in most cases from the carotid artery, and muscle (1-5 grams) were taken before the injection and immediately put into acetone-free methyl alcohol.⁷ The blood was drawn with pipette and needle as suggested by Folin and Denis.⁸ An incision was made in the midline of the abdomen, and the celiac axis and superior and inferior mesenteric arteries ligated. A mass ligature was then tied around the hepatic artery, portal vein and bile-duct, and finally the renal vessels were dissected out and tied. The entire operation consumed in the majority of instances from six to fifteen minutes. In the tables these cats are designated as "operated." Samples of the liver were taken at this stage in some cases. The abdominal wall was then closed with sutures or clamps, and intravenous injection of the amino-acid (dissolved in 50 cc. of Ringer's solution) begun, preceded by 5 or 10 cc. of Ringer's solution. The injection was made from a burette by way of a glass cannula, usually into the external jugular vein. Unless otherwise stated, the muscle samples were taken from the gracilis. In most cases we used the right carotid artery and the right gracilis muscle for the first samples, the left for the second. The second samples were taken at the end of the experiment in the same way as before. Samples were sometimes taken during the experiment in addition. The controls were treated similarly, with the exception that only the renal vessels were ligated.

Analyses of total non-protein nitrogen and urea were made by the methods of Folin and Denis,⁹ with slight modifications in the case of tissue:

Muscle samples were cut into small pieces immediately after removal, and immersed in about 30 cc. of methyl alcohol in a 100-cc. Erlenmeyer

⁷ Negative test with Scott-Wilson reagent.

⁸ This *Journal*, xi, p. 527, 1912.

⁹ *Loc. cit.*

flask. Twenty-four hours later the alcohol is poured off into a 100-cc. volumetric flask, and the muscle ground in a mortar with purified quartz sand, then triturated with 10 cc. of methyl alcohol and transferred back to the Erlenmeyer flask with a spoon, the contents of the mortar washed into the flask with three 10-cc. portions of alcohol and the whole extracted another twenty-four hours. The contents of the Erlenmeyer flask were then filtered into the volumetric flask containing the first extract, and the residue washed on the filter with measured portions of alcohol, to within 1 cc. of the mark. Ten drops of saturated alcoholic solution of ZnCl_2 were added and the volume made up to the mark. After standing thirty minutes or more the extract was filtered through a dry filter into a clean, dry 100-cc. Erlenmeyer flask, and tightly stoppered. The liver samples were treated in the same way, but without the use of sand. Samples from the same experiment were always treated exactly alike and at the same time.

All analytical figures are given in terms of milligrams of nitrogen per 100 grams of blood or tissue.

Ammonia determinations on a considerable number of the tissue extracts have convinced us that none of the variations in the urea content of tissue during our experiments can be attributed to ammonia. We omit the figures inasmuch as we have reason to believe that they do not represent the true values. We are working on this point now. Ammonia determinations on the blood in typical cases by the method of Folin and Denis¹⁰ have likewise shown that this plays no part in the changes in urea content recorded. These results are quite different from those of Salaskin and Kowalewsky.¹¹

The accumulation of urea without injection of amino-acid.

A few preliminary experiments were undertaken to determine the degree of urea accumulation occurring in the course of two or three hours after ligation of the renal vessels, with nothing injected into the circulation.

There is seen to be a slight rise in the urea content of the blood. The results are similar to those of Kaufmann,¹² who observed in most cases a slight increase in the urea content of the blood in dogs during the first hour after ligation of the aorta and the vena cava above the diaphragm. Kaufmann's experiments have

¹⁰ *Loc. cit.*

¹¹ *Zeitschr. f. physiol. Chem.*, xlii, p. 410, 1904.

¹² *Arch. de physiol.*, xxvi, p. 531, 1894.

TABLE 1.
No injection.

CAT NO.	WEIGHT	DURATION	BLOOD				MUSCLE				REMARKS
			Total non-protein nitrogen		Urea nitrogen		Total non-protein nitrogen		Urea nitrogen		
			Before	After	Before	After	Before	After	Before	After	
16	kilos	hrs.	52	52	34	37					Control
44	2.30	3	61	64	38	43	283*	298	39	39	Control
20	2.45	2	79	88	40	42	282	316	54	54	Operated
24	1.70	3	41	51	26	27	276	289	33	34	Operated

* Pectoralis.

heretofore constituted the only incontestable demonstration of urea formation outside the liver. His observation is confirmed by the above figures. The experiments to follow, in which amino-acids were injected intravenously, show the same thing more clearly, the increase in urea being far beyond the limits of error.

Urea formation from amino-acids with the liver excluded from the circulation.

Our earlier experiments were extended over a period of three hours, the injection occupying the first one hundred minutes of this. The "operated" animals of these experiments were moribund even before the injection was completed. The blood pressure was very low, the heart beat could hardly be felt, and the mucous membranes were bloodless. The blood taken from the artery at the end of the experiment in most cases was very dark, and was never of normal color. The results follow (Table 2).

The increase in urea in the blood in these experiments is on the whole less marked in the "operated" animals than in the controls, in the muscle much less so. The results show, however, much more definitely than any now on record, that urea formation from amino-acids does occur outside the liver. The absence of any increase in urea in the muscle in Cats 28 and 35, as well as the slightness of the increase in total non-protein nitrogen, is probably

TABLE 2.

5 grams glycocoll in 50 cc. Ringer's solution injected intravenously during 1 hour 40 minutes. Samples taken before injection, and 1 hour 20 minutes after. Length of experiments, 3 hours.

CAT NO.	WEIGHT	BLOOD				MUSCLE				REMARKS
		Total non-protein nitrogen		Urea nitrogen		Total non-protein nitrogen		Urea nitrogen		
		Before	After	Before	After	Before	After	Before	After	
	kilos									
22	1.50	35	130	19	40	262	288	26	42	Control
43	3.29	47	111	36	70	246	264	48	69	Control
15	1.93	52	144	29	42					Operated
28	2.76	79	210	55	71	258	286	54	50	Operated
35	2.10	49	168	21	31	258	273	33	33	Operated
37	1.82	59	198	38	53	317	443	53	58	Operated
38	3.28	38	121	20	30	228*	271	37	41	Operated
39	2.45	29	145	12	23	238†	301	14	18	Operated

* Pectoralis.

† Teres major.

due to the fact that we used here muscle samples weighing 5 grams, as compared with about 2 grams in the others of this series. The extraction in the former case is less complete.

Later experiments lasted one hour, the amino-acid being injected during the first fifty minutes. In these animals there was no pronounced fall in blood pressure. The blood, of normal color, flowed into the pipette, as the final sample was being drawn, at a rate indistinguishable from that at the beginning. As is to be expected, urea formation is in these cases less marked in the controls than in the preceding experiments, especially with respect to its accumulation in muscle.

Taking blood and muscle together, there is no essential difference between the "operated" animals and the controls. Urea formation from the injected amino-acid occurs in both to approximately the same extent.

For the sake of demonstrating the absence of circulation through the liver in the "operated" animals, a gelatine mass, colored with lead chromate, was injected into the aorta and the superior mesenteric vein of Cat 35 after the experiment. The liver showed no yellow color, nor could a qualitative test for lead be obtained in an

TABLE 3.

5 grams glycocoll in 50 cc. Ringer's solution injected intravenously during 50 minutes. Samples taken before injection, and 10 minutes after. Length of experiments, 1 hour.

CAT NO.	WEIGHT	BLOOD				MUSCLE				REMARKS
		Total non-protein nitrogen		Urea nitrogen		Total non-protein nitrogen		Urea nitrogen		
		Before	After	Before	After	Before	After	Before	After	
	<i>kilos</i>									
46	3.06	89	131	71	81	299	314	82	84	Control
47	4.26	36	85	22	34	206	212	27	30	Control
48	2.80	43	128	27	45	264	282	40	42	Operated
49	3.37	39	120	22	30	248	286	35	40	Operated

TABLE 4.

2.6 grams *dl*-alanine in 50 cc. Ringer's solution injected intravenously during 50 minutes. Samples taken before injection, and 10 minutes after. Length of experiments, 1 hour.

CAT NO.	WEIGHT	BLOOD				MUSCLE				REMARKS
		Total non-protein nitrogen		Urea nitrogen		Total non-protein nitrogen		Urea nitrogen		
		Before	After	Before	After	Before	After	Before	After	
	kilos									
59	3.14	54	74	37	54	271	281	51	53	Control
56	2.32	37	126	21	29	300	315	33	38	Operated
57	2.46	29	120	16	28	293	330	40	44	Operated

incinerated sample. It is possible, however, that small branches to the capsule, etc., were permeable before death, and still were not reached by the injection mass. In a few typical experiments total non-protein nitrogen determinations were made on liver samples before and after the injection of amino-acid, the samples being taken at about the same time as those of muscle. The results show definitely that no amino nitrogen reached the livers of the "operated" cats. The observed urea formation in these animals is therefore independent of the liver.

TABLE 5.
Liver analyses.

CAT NO.	INJECTED	TOTAL NON-PROTEIN NITROGEN IN LIVER		REMARKS
		Before	After	
43	5 gm. glycocoll.....	143	269	Control
54	2.6 gm. alanine.....	174	230	Control
37	5 gm. glycocoll.....	189	186	Operated
62	5 gm. alanine.....	178	180	Operated

While this work was in progress there appeared a series of papers by Van Slyke and Meyer,¹³ in part concerned with the distribution of injected amino-acids and with the site of urea formation. They observed that amino-acids, injected intravenously, accumulate at first to a greater extent per unit of mass in the liver than in muscle, and that this excess in the liver disappears in the course of a short time, the excess in the muscle remaining constant. The following figures, calculated from data given elsewhere in this paper, point in the same direction.

TABLE 6.
Accumulation of amino-acid in liver and muscle.

CAT NO.	AMINO-ACID INJECTED	AMINO N INJECTED	TIME AFTER INJECTION	INCREASE IN TOTAL NON-PROTEIN NITROGEN	
				Muscle	Liver
		<i>mgm.</i>	<i>min.</i>		
43	Glycocoll.....	933	80	18	126
54	Alanine.....	409	5 to 9	38	66
			60	57	20

Van Slyke and Meyer, however, neglect the fact that the total weight of muscle in the body is at least eight times that of the liver. The total accumulation in muscle, in their experiments as well as in ours, is much greater than in the liver. The figures given above indicate that this relatively greater initial accumulation in the liver depends upon the amount of amino-acid injected. That it occurs at all during the normal process of amino-acid absorption from the digestive tract remains entirely undemonstrated, and

¹³ This *Journal*, xvi, pp. 197, 213, 1913.

appears unlikely from data at present available. There is nothing remarkable in the fact that the liver should get rid of its accumulated amino nitrogen first, as occurs with glycogen. The storage of carbohydrate in the liver seems indeed, apart from temporal relations, to be an analogous phenomenon, and yet there is no reason for believing, upon this basis, that the liver is the sole or chief site of carbohydrate cleavage. We can see, therefore, no reason for assuming, from the manner in which injected amino-acids are distributed in the body, that urea formation from this source takes place in particular in the liver.

The following experiment shows much the same thing as the analogous ones of Van Slyke and Meyer.

TABLE 7.

Cat 54. Weight, 3.01 kilos. 2.6 grams alanine in 50 cc. Ringer's solution injected into the right femoral vein in ten minutes.

	BLOOD			MUSCLE*		
	Total non-protein nitrogen	Urea nitrogen	Difference	Total non-protein nitrogen	Urea nitrogen	Source
Before injection....	36	23	13	225	38	Right gracilis
5 minutes after injection.....	88	28	60	263		Right semi-membranosus
1 hour after injection.....	55	35	20	282	50	Left gracilis
3 hours after injection.....	55	44	11	271	51	Left semi-membranosus

* 1.6 gms. per sample.

	LIVER	
	Total non-protein nitrogen	Urea nitrogen
Before injection.....	174	42
9 minutes after injection.....	230	
1 hour 10 minutes after injection.....	194	54
3 hours after injection.....	199	51

The main point in this experiment which we wish to bring out is the close parallelism in the degree of accumulation of urea in muscle and liver. In another similar experiment, the urea nitrogen increased in the muscle from 48 to 69 mgm., in the liver from 50 to 71 mgm. per 100 grams. There is obviously nothing here to indicate a special site of urea formation.

As an additional control, to rule out the slight possibility that the increase in urea found after amino-acid injection in the "operated" animals may have been due to changes in concentration or distribution, a solution of sodium chloride having potentially twice the osmotic pressure of the most concentrated amino-acid solution used was injected into a cat, after ligation of the blood supply to the liver, etc., as before.

TABLE 8.

Cat 63. Weight 3.05 kilos. 4.0 gm. NaCl in 50 cc. Ringer's solution injected intravenously, at the rate of 1 cc. per minute.

	BLOOD		Muscle	
	Total non-protein nitrogen	Urea nitrogen	Total non-protein nitrogen	Urea nitrogen
Before injection.....	77	59	298	60
10 minutes after injection.....	71	57	324	62

SUMMARY AND CONCLUSIONS.

1. Direct, unassailable evidence that the liver is the chief site of urea formation from amino-acids does not exist.
2. The accumulation of urea per unit of mass in blood and tissues after intravenous administration of amino-acids, in the experiments above recorded, is as great (in the absence of shock) when the liver and other abdominal viscera are excluded from the circulation as when they are in their normal relations.
3. We have therefore adduced direct evidence that the liver is not the chief site of urea formation from amino-acids.

We wish to express our gratitude to Prof. Otto Folin for permission to carry on this work in his laboratory, and for his advice and interest.

A VOLUMETRIC METHOD FOR THE ESTIMATION OF TOTAL SULPHUR IN URINE.

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During the last ten years, it has been shown that the quantitative determination of sulphur could be accomplished not only by weighing it as barium sulphate, but also, with the same degree of accuracy, by precipitating it as benzidine sulphate, and titrating the sulphuric acid with standard alkali. It is known that when a soluble salt of benzidine, *e.g.*, benzidine hydrochloride, is mixed with a solution containing sulphuric acid or soluble sulphates, a precipitate is formed consisting of benzidine sulphate. This reaction, carried out under certain conditions which have been determined by Müller,¹ Raschig, Friedheim and Nydegger, and others, is quantitative. The compound, benzidine sulphate, behaves, in water, like a mixture of benzidine and sulphuric acid, and this work² was begun with the object of ascertaining whether in the estimation of total sulphur in urine according to Benedict,³ it would be possible to substitute the titration of the sulphuric acid in the benzidine sulphate precipitate by standard alkali for the gravimetric determination of barium sulphate, now universally practiced.

¹ W. Müller: *Ber. d. deutsch. Chem. Gesellsch.*, xxxv, p. 1587, 1902; Müller and Durkes: *Zeitschr. f. anal. Chem.*, xlii, p. 477, 1903; F. Raschig: *Zeitschr. f. angew. Chem.*, 1903; Friedheim and Nydegger: *ibid*, 1907.

² After this work was completed our attention was called to the somewhat similar work of O. Rosenheim and J. C. Drummond: *Biochem. Journ.*, viii, April, 1914. These authors applied the benzidine method for the determination of inorganic and ethereal sulphates in urine, with good results.

³ This *Journal*, vi, p. 363, 1909.

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Two distinct disadvantages were immediately encountered: First, the amount of sulphuric acid derivable from 10 cc. of urine is so small as to make its titrimetric estimation uncertain. The use of larger amounts of urine makes the procedure less simple than the original method of Benedict. Second, the end point in the titration of the sulphuric acid in a benzidine sulphate precipitate is not reached at once, being obtained only after repeated boilings and additions of alkali.

To overcome these difficulties, we have devised a method for estimating the benzidine in the precipitate of benzidine sulphate, rather than the sulphuric acid. We have found that benzidine, in acid medium, reduces potassium permanganate quantitatively, with the instant production of a canary-yellow soluble product which, on further addition of permanganate, is converted into a colorless substance. Further, a small amount of benzidine reduces a relatively large volume of $\frac{N}{10}$ potassium permanganate.

In applying this reaction to the quantitative estimation of the benzidine in benzidine sulphate, certain conditions must be observed. Towards the end of the titration, the pink color of the permanganate disappears more and more slowly, and this enables us to obtain a definite end point, which, in our work, is a distinct pink lasting for twenty seconds. Variations in temperature influence the titration: it is therefore necessary to titrate at a certain definite temperature. If the benzidine sulphate is dissolved in 200 cc. of water at room temperature, and to it are added 20 cc. of sulphuric acid (conc.), then the resulting temperature is taken as a standard for all titrations. It was noticed that benzidine sulphate, in suspension, was oxidized irregularly, and therefore it was necessary to have the benzidine sulphate in solution before titrating. This is possible, since a small amount of benzidine sulphate is soluble in boiling water. Once brought in solution, it remains so both after cooling and after addition of sulphuric acid (conc.). For the sake of rapid filtration and thorough washing of the benzidine sulphate, we use strong suction, without fear of compressing the precipitate. The addition of a small amount of sodium hydroxide greatly facilitates the process of dissolving the benzidine sulphate, even if the latter is suspended in the form of compressed lumps.

It was found that the precipitation of benzidine sulphate was

markedly interfered with by the presence of free hydrochloric acid; a series of experiments showed that the more free hydrochloric acid there was present, the less benzidine sulphate was formed. Inasmuch as we were dealing with small amounts of benzidine sulphate, it was essential to reduce the amount of free hydrochloric acid to a minimum. This was accomplished by neutralizing the clear hydrochloric acid solution with sodium hydroxide (10 per cent), until one drop produced a precipitate of copper hydroxide, which was then redissolved with one drop of hydrochloric acid (1: 4). If the solution is neutralized and acidified carefully, the benzidine sulphate precipitates quantitatively.

The speed and accuracy of the work are increased by the use of asbestos filters, a scheme devised by us for vacuum filtration of precipitates, which are subsequently analyzed by volumetric methods. The preparation of an asbestos filter for small precipitates is as follows:—Fit a 4 cm. funnel to a 250 cc. filtering flask; fill the funnel two-thirds full with absorbent cotton, moisten it with water, and press the cotton down, forming a flat surface. Pour a suspension of asbestos on the cotton, sufficient to form a thin layer of asbestos, which is easily separated from the cotton when the filtration is finished. With such filters, a strong suction may be safely used.⁴

The $\frac{N}{10}$ potassium permanganate was standardized by Sørensen's sodium oxalate method. A weak sulphuric acid solution was analyzed gravimetrically, four results agreeing closely; 1 cc. of this sulphuric acid contained 0.000166 gram of sulphur. In order to establish the value of 1 cc. of $\frac{N}{10}$ potassium permanganate in terms of sulphur, 5 and 10 cc. respectively, of the sulphuric acid were treated with benzidine hydrochloride, the precipitate filtered, washed, and titrated. Below are the results of sixteen such analyses, and their average value, we think, may safely be taken as a basis for the proposed method of analysis.

⁴ We have used a larger form of filter to advantage in the filtration of ammonium urate obtained in the Folin-Shaffer method for uric acid, and in the filtration of ammonium phosphomolybdate in Neumann's method for the determination of phosphorus. In each case, a 6 cm. funnel was used, and the filtering and washing was accomplished with great ease and thoroughness; good duplicates were obtained, the asbestos present having no influence on the end point in the titration.

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For 5 cc. H_2SO_4 were required 8.35, 8.48, 8.39, 8.39, 8.44, 8.44, 8.25, 8.49; average, 8.40 cc. $\frac{N}{10}$ KMnO_4 . For 10 cc. H_2SO_4 were required 16.83, 16.80, 16.82, 16.60, 16.64, 16.73, 16.94, 16.83; average 16.77 cc. KMnO_4 .

Taking the average of these analyses we find that 1 cc. of $\frac{N}{10}$ potassium permanganate corresponds to 0.000099 gram of sulphur.

The method, which we propose for the determination of total sulphur in urine, is as follows: To 2 cc. of urine, in an 8 cm. porcelain dish, add 0.5 cc. of Benedict's reagent, and evaporate to dryness on the water bath. Heat the dish carefully over a small flame, till the contents are black, and then heat to redness for only two minutes. Cool, add 2 cc. of hydrochloric acid (1:4), and warm. The clear solution is neutralized with sodium hydroxide (10 per cent), and again acidified with one drop of hydrochloric acid (1:4); 25 cc. of a solution of benzidine hydrochloride⁵ are added, with stirring, the resultant precipitate allowed to stand fifteen minutes and then filtered off on an asbestos filter. The precipitate of benzidine sulphate is quantitatively transferred to the filter by means of the filtrate, and washed with 5 cc. of cold water, drop by drop. The filter is put into a 500-cc. Erlenmeyer flask, 1 cc. of sodium hydroxide (10 per cent) and 200 cc. of water are added, the suspension boiled for five minutes, and then cooled to room temperature; 20 cc. of sulphuric acid (conc.) are now added, and the warm solution immediately titrated with $\frac{N}{10}$ potassium permanganate, till a distinct pink coloration is obtained, which should last for twenty seconds. In titrating, at first add only about 0.5 cc. at a time, and towards the end, only 2 drops at a time, waiting till the color disappears before further addition of potassium permanganate. As the titration progresses, it will be noticed that the yellow color gradually disappears, the solution turning practically colorless. It is at this stage of the titration, that care should be taken in adding only 2 drops of permanganate at a time.

If the urine is concentrated, and contains a large amount of sulphur, 1 cc. may be taken for analysis; if very diluted urines, or those of small animals are analyzed, 5 cc. or more may be taken.

In Benedict's method, we have a comparatively quick and accurate procedure for the determination of total sulphur in the urine.

⁵ 6.7 gm. of benzidine (Merck reagent) are put into a 1-liter flask, 29 cc. of hydrochloric acid (sp. gr., 1.12) added, and the solution diluted up to the mark.

We had the opportunity, in our laboratory, of making hundreds of these determinations according to the original process. We also compared Benedict's method with Folin's⁶ peroxide method, and with Denis'⁷ modification of Benedict's method. It was found that all three methods gave concordant results.

The first part of the method we propose is similar to Benedict's. The gravimetric determination of barium sulphate, however, is substituted by a volumetric oxidation procedure. Since it was found possible to estimate amounts of sulphur smaller than have ever before been determined, and since 2 cc. of urine instead of 10 cc. may be analyzed Benedict's method, with our modification, becomes extremely convenient and rapid.

Below are the results of analyses by the new method, compared to those obtained with Benedict's procedure.

Urine A. By Benedict's method two analyses, each of 10 cc., showed 0.0368 grams and 0.0368 gram of barium sulphate; in a 24-hour sample, this is equal to 1.0120 grams of sulphur.

By the volumetric method, the following results were obtained:

5 cc. urine analyzed.

$\frac{N}{10}$ KMnO ₄	S	S in 24 hrs.
cc.	gm.	gm.
25.30	0.002505	1.0020
25.10	0.002488	0.9952
25.20	0.002497	0.9988

2 cc. urine analyzed.

10.08	0.000998	0.9980
10.08	0.000998	0.9980
9.88	0.000979	0.9790
9.97	0.000987	0.9870
10.08	0.000988	0.9980
10.08	0.000988	0.9980

Urine B. By Benedict's method two analyses, each of 10 cc., showed 0.0395 gram and 0.0400 gram of barium sulphate; in a 24-hour sample, this is equal to 1.0920 grams of sulphur.

⁶ Folin: *Journ. Amer. Chem. Soc.*, xxi, 1908.

⁷ Denis: *This Journal*, viii, 1910.

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The volumetric method yielded the following results:

2 cc. urine analyzed.

$\frac{N}{10}$ KMnO ₄	S	S in 24 hrs.
cc.	gm.	gm.
10.78	0.001067	1.0670
10.85	0.001074	1.0740
10.94	0.001082	1.0820
10.92	0.001081	1.0810
10.82	0.001071	1.0710
10.80	0.001069	1.0690
10.70	0.001059	1.0590

Urine C. By Benedict's method three analyses, each of 10 cc., showed 0.0195 grams, 0.0195 grams and 0.0197 gram of barium sulphate; in a 24-hour sample, this is equal to 0.6725 gram of sulphur.

The volumetric method yielded the following results:

5 cc. urine analyzed.

$\frac{N}{10}$ KMnO ₄	S	S in 24 hrs.
cc.	gm.	gm.
13.78	0.001363	0.6815
13.92	0.001377	0.6885
13.82	0.001367	0.6835
13.88	0.001373	0.6865

2 cc. urine analyzed.

5.48	0.0005425	0.6780
5.44	0.0005380	0.6730
5.44	0.0005380	0.6730

A glance at the above tables will show that our method gives results similar to Benedict's. An analogous procedure for the estimation of different sulphur fractions in the urine, and also other applications of the new method are being worked out.

SUMMARY.

1. A volumetric method is proposed for the determination of total sulphur in urine.

2. This method has for its basis, the estimation of benzidine in the precipitate of benzidine sulphate, rather than the sulphuric acid.

3. The procedure occupies only one-fifth of the time required by the Benedict method.

4. Very small amounts of sulphur may be accurately determined by the proposed method.

ON VICINE.

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New York.)

(Received for publication, June 2, 1914.)

Vicine was discovered by Ritthausen¹ who found that the substance could be hydrolyzed into a hexose and into a basic substance which was named by him divicine. The exact nature of the base and the configuration of the sugar were not recognized by the discoverer of the substance. Schulze and Trier,² on the basis of theoretical considerations, were the first to give expression to the assumption that vicine had the structure of a pyrimidine glucoside. Very recently T. B. Johnson,³ and Johnson and Johns⁴ have added both theoretical and experimental evidence in support of the same assumption. These authors, following the directions of Traube, prepared two pyrimidine bases: 4-6-dioxy-2-5-diaminopyrimidine and 2-6-dioxy-4-5-diaminopyrimidine. Comparing the properties of the two synthetic bases with the properties of divicine as described by Ritthausen the authors were led to the belief that divicine was identical with 2-6-dioxy-4-5-diaminopyrimidine.

At the time of the publication of the T. B. Johnson's first article the present work was already in progress, and we then entered into an agreement with Professor Johnson to postpone further experiments until after the publication of the further two articles by Johnson and Johns. Since the articles of Johnson and Johns

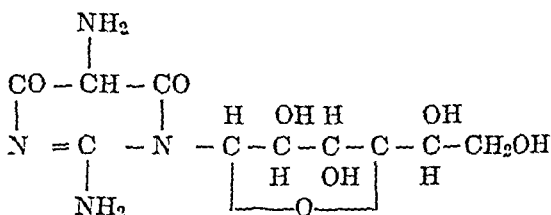
¹Ritthausen: *Journ. f. prakt. Chem.*, ii, p. 336, 1870; vii, p. 374, 1873; *Ber. d. deutsch. chem. Gesellsch.*, ix, p. 301, 1876; *Journ. f. prakt. Chem.*, xxiv, p. 202, 1881; *Ber. d. deutsch. chem. Gesellsch.*, xxix, pp. 894 and 2108, 1896; *Journ. f. prakt. Chem.*, lix, pp. 480 and 487, 1899.

²Schulze and Trier: *Zeitschr. f. physiol. Chem.*, lxx, p. 143, 1910.

³Johnson: *Journ. Amer. Chem. Soc.*, cxxvi, p. 337, 1914.

⁴Johnson and Johns: *Journ. Amer. Chem. Soc.*, xxxvi, pp. 545 and 970, 1914.

do not exhaust the subject of the structure of vicine, I feel justified in publishing the results of the present investigation, which furnish information on the principal points in the structure of the nucleoside. The results point to the conclusion that vicine is composed of 4-6-dioxy-2-5-diamino-pyrimidine combined in glycosidic union with *d*-glucose. Regarding the place of the union between sugar and base the information is not yet absolutely definite. The fact that both vicine and divicine seem to give off about 50 percent of their nitrogen in form of nitrogen gas on treatment with nitrous acid seems to indicate that the union is not through the medium of one of the amino-groups. Hence one may be justified in representing vicine provisionally by the following graphic formula:



This conception, however, is not in complete harmony with all of its known properties.

It seems to be contradicted by the fact that vicine does not give the color test with molybdic acid. Divicine, on the other hand, does give a positive color test with the same reagent, and Johnson and Johns reached the conclusion that the presence of a free amino-group in position 5 results in a positive molybdic test.

The nature of the sugar is based on properties of the osazone: M.P. = 205°C. and rotation in a 0.5 dm. tube (0.200 gram in 10 cc. of Neuberg's pyridine-alcohol mixture) was: Initial, -0.49° ; equilibrium, -0.39° . The rotation of a glucosazone under the same condition was identical. On oxidation with nitric acid the sugar formed saccharic acid, which was identified as the acid potassium salt.

The base was recognized as 4-6-dioxy-2-5-diamino-pyrimidine on the following grounds. The 4-6-dioxy-2-5-diamino and 2-6-dioxy-4-5-diamino-pyrimidines were prepared by the method of Traube. These two bases differ one from another in the following three properties. The first base forms a sulphate which crystal-

lizes without or with one molecule of water of crystallization, on treatment with nitrous acid in the Van Slyke apparatus it gives rise to nitrogen gas in a quantity equal to about 80 per cent of that required for two primary amino-groups, and on condensation with urea, under the conditions given by Johnson and Johns, it does not give rise to uric acid. The second base forms a sulphate, crystallizing out of water with one and one-half molecules of water according to Traube; the sulphate prepared in course of this work contained about two and one-half molecules of water; with nitrous acid in the apparatus of Van Slyke it forms a very insignificant quantity of nitrogen gas (perhaps due to impurity). It is interesting to note that 2-6-dioxy-4-amino-pyrimidine under the same conditions forms nitrogen gas in quantity equal to 33 per cent of its total nitrogen.) Further, as shown by Johnson and Johns, the base condenses with urea to form uric acid.

Divicine, in most of its properties, comes closer to 4-6-dioxy-2-5-diamino-pyrimidine than to its isomer. True, it was difficult to obtain uniform analytical figures for the sulphate of divicine, a fact already observed by Ritthausen, but, of many samples analyzed all possessed more resemblance to the first of the two bases. In its behavior towards nitrous acid and towards urea it was decidedly different from 2-6-dioxy-4-5-diamino-pyrimidine, on the contrary it showed in this respect a striking resemblance to the isomer.

EXPERIMENTAL PART.

Preparation of vicine was facilitated by slight modifications in the original Ritthausen method. The vetch meal was extracted with 5 per cent sulphuric acid for three hours, at the end of which the mixture was neutralized with barium hydrate solution, filtered, and the filtrate precipitated with a 10 per cent solution of mercuric sulphate in 5 per cent sulphuric acid. The mixture again was neutralized with barium hydrate solution. The precipitate was allowed to settle and was then removed by filtration, washed and suspended in water to which an excess of barium carbonate was added. The precipitate was finally freed from mercury by means of hydrogen sulphide and the filtrate from mercuric sulphide concentrated to a small volume, first on the water bath and finally under diminished pressure. To the concentrated solution 95 per

cent alcohol was added as long as it formed a precipitate and the mixture was brought to a boil on water bath. It was then filtered, and the filtrate concentrated to a very small volume under diminished pressure. Analytically pure and colorless vicine crystallized out on cooling. Once recrystallized the substance had a M. P. = 242°C . (uncorrected) and the following optical rotation:

0.300 gram of substance in 3 cc. of water had the weight of 3.7934 grams. The rotation in 1 dm. tube in pure yellow light was $\alpha = -0.93^{\circ}$. Hence

$$[\alpha]_D^{25} = -11.7^{\circ}$$

Nitrogen distribution in vicine.

0.1000 gram of the substance contained 0.01736 gram of nitrogen.

0.1000 gram of a second sample contained 0.0174 gram of nitrogen.

Amino nitrogen estimation. 0.050 gram were used in the first experiment. The conditions of experiment were the usual indicated by Van Slyke. In forty minutes the evolution of nitrogen reached its maximum. It was 8 cc. of gas at 30°C ., 769 mm., or amino N = 50 per cent of the total nitrogen.

It was thought that the high amino-nitrogen value was conditioned by the fact that the nucleoside was hydrolyzed in course of the experiment. Hence the acidity of the nitrite mixture was diminished by employing one part of acetic acid to 5 parts of the sodium nitrite solution.

0.050 gram of the vicine evolved the following values of nitrogen gas:

15 minutes..... 5.4 cc.

'25 minutes..... 8.8 cc.

40 minutes. (No further changes)..... 8.8 cc.

Correction, 0.8 cc. Hence final volume = 8 cc. of N at 26°C . and 760 mm., or amino N = 49.98 per cent of total nitrogen.

It is realized that other methods will have to be employed to determine the number of free NH_2 groups in the vicine molecule.

Preparation of divicine sulphate and its composition. Five-gram lots of vicine were taken up in 25 cc. of a 20 per cent solution of sulphuric acid and placed in a boiling water bath. A crystalline deposit soon appeared in the solution. As soon as the deposit reached its maximum the heating was discontinued, the reaction product cooled and the precipitate filtered and dried in the air over night. Before analysis the substance was recrystallized out of 10 per cent sulphuric acid.

0.1176 gram of the substance gave 0.1066 gram CO_2 and 0.4100 gram H_2O .

0.1000 gram of the substance gave 0.0574 gram BaSO_4 .

0.050 gram of the substance gave 0.0139 gram nitrogen by Kjeldahl method.

0.050 gram of the substance dissolved in 5 cc. of 10 per cent solution of sulphuric acid gave 0.0064 gram amino N in the Van Slyke apparatus.

Analysis of the sulphate of 2-6-dioxy-4-5-diamino-pyrimidine. The substance was prepared according to the method of Traube.

Sample I. 0.1181 gram of substance gave 0.0979 gram CO₂ and 0.0466 gram H₂O.

0.1000 gram of substance gave 0.0518 gram BaSO₄.

0.050 gram of substance gave 0.1345 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in 10 per cent solution of sulphuric acid gave 0.0007 gram nitrogen by Van Slyke method.

Sample II. 0.050 gram of substance gave 0.0135 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in a 10 per cent solution of sulphuric acid gave 0.0009 gram nitrogen in Van Slyke apparatus.

Analysis of 2-6-dioxy-4-amino-pyrimidine.

0.050 gram of substance gave 0.0165 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in a 10 per cent solution of sulphuric acid gave by Van Slyke method 0.0051 gram of nitrogen. Hence, ratio of

$$\frac{\text{Amino N}}{\text{Total N}} = 1:3.$$

Analysis of 4-6-dioxy-2-5-diamino-pyrimidine. The base was prepared by the two methods of Traube. The first sample by reducing with a current of hydrogen sulphide the isonitrosomalonylguanidine in hydrochloric acid solution. The hydrochloride of the base was then transformed into the sulphate. The hydrochloride was employed for the estimation of the amino-nitrogen since the sulphate was found too insoluble for the purpose.

The second sample of the base was prepared by the sulphuric acid process, in which the isolation of the isonitrosoderivative is omitted.

The composition of the sulphates obtained by the two different processes was identical.

0.050 gram of the hydrochloride gave 0.01414 gram of nitrogen by the Kjeldahl method.

0.050 gram of the same substance gave 0.00505 gram of nitrogen in Van Slyke's apparatus.

0.050 gram of the sulphate obtained from the hydrochloride gave 0.01335 gram of nitrogen by the Kjeldahl method.

0.100 gram of the substance gave 0.0582 gram BaSO₄.

0.050 gram of the second sample of the sulphate gave 0.0142 gram of nitrogen by the Kjeldahl method.

0.100 gram of the substance gave 0.0598 gram of BaSO_4 .

Analysis of results.

Calculated for 2-6-dioxy-4-5-diamino-pyrimidine ($\text{C}_4\text{H}_6\text{N}_4\text{O}_2$) $_2$ H_2SO_4 + $2\frac{1}{2}\text{H}_2\text{O}$:			Found:
C.....	22.50		22.61
H.....	4.50		4.42
N.....	27.00	(I) 26.90 (II) 27.00	
NH_2N	13.50		(trace)
H_2SO_4	22.90		22.11

Calculated for 4-6-dioxy-2-5-diamine ($\text{C}_4\text{H}_6\text{N}_4\text{O}_2$) $_2$ H_2SO_4 + H_2O :			Found:
N.....	28.00	(I) 27.80 (II) 27.70	
H_2SO_4	24.50	(I) 24.28 (II) 24.45	

Calculated for ($\text{C}_4\text{H}_6\text{N}_4\text{O}_2$) HCl + H_2O :			Found:
N.....	28.49		28.28
NH_2N	14.24		11.30

Calculated for divicine sulphate ($\text{C}_8\text{H}_{12}\text{N}_8\text{O}_2$) H_2SO_4 + H_2O :			Found:
C.....	24.00		24.72
H.....	4.00		3.90
N.....	28.00		27.80
NH_2N	14.00		12.80
H_2SO_4	24.50		24.16

Condensation with urea.

2-6-dioxy-4-5-diamino-pyrimidine. 0.5 gram of the free base and 0.5 gram of urea were heated in a small tube at 160 to 170°C. for one hour. The reaction product was dissolved by the aid of very little alkali, and acidulated by means of hydrochloric acid. A very light yellow precipitate formed, which was carefully washed and dried. With ammoniacal silver solution it formed a precipitate insoluble in excess of ammonia.

0.050 gram of the substance gave 0.0165 gram nitrogen by Kjeldahl method.

Calculated for $\text{C}_8\text{H}_{12}\text{N}_8\text{O}_2$:			Found:
N.....	33.33		33.00

In a second experiment 1 gram of the sulphate was intimately triturated with 1.5 grams of urea and treated as in the first experi-

ment. The final product had the same appearance as in the first experiment.

0.050 gram of the substance gave 0.01659 gram nitrogen by Kjeldahl method.

	Calculated for $C_5H_4N_4O_3$:	Found:
N.....	33.33	33.18

4-6-dioxy-2-5-diamino-pyrimidine, and divicine on condensation with urea under identical conditions did not form uric acid.

Analysis of carbohydrate. 10 grams of vicine were hydrolyzed in the usual way. The divicine sulphate was removed by filtration. The filtrate diluted to 300 cc. and the sulphuric acid removed by means of lead carbonate, and the lead by means of hydrogen sulphide. A part of the solution was used for the preparation of the phenyl osazone. It was prepared in the usual way. For analysis it was recrystallized out of an alcohol-acetone mixture. The melting point = 205°C .

0.1187 gram of the substance gave 0.2635 gram CO_2 and 0.0652 gram H_2O .

	Calculated for $C_{18}H_{22}N_4O_4$:	Found:
C.....	60.30	60.54
H.....	6.14	6.15

0.200 gram of the substance dissolved in a mixture of 2 cc. pyridine and 3 cc. of alcohol rotated in a 0.5 dm. tube in yellow light, $\alpha = -0.49^{\circ}$ in twenty-four hours, $\alpha = -0.39^{\circ}$.

0.200 gram of glucosazone under the same condition, $\alpha = -0.49^{\circ}$; in twenty-four hours, $\alpha = -0.39^{\circ}$.

The remaining part of the solution was oxidized with nitric acid and from the reaction product the acid potassium salt of saccharic acid obtained. For analysis it was once recrystallized out of water.

0.100 gram of the substance gave 0.0354 gram K_2SO_4 .

	Calculated for $C_6H_5O_5K$:	Found:
K.....	15.74	15.90

THE IODINE CONTENT OF TUBERCULOUS TISSUES.

By PAUL A. LEWIS AND ROBERT B. KRAUSS.

(From the Henry Phipps Institute of the University of Pennsylvania.)

(Received for publication, June 2, 1914.)

During the past two years we have prepared a number of compounds of iodine with the dyes Trypan-red¹ and Trypan-blue and have studied them in animal experiments with the idea that the well-known action of these dyes as vital stains might be used to distribute physiologically active iodine to tuberculous tissue. A number of the dye-stuff compounds with iodine are vital stains with much the same factors of distribution as the original dyes. As in the case of the mother substances many of these iodized dyes show considerable tendency to concentrate in the experimental tuberculous tissues of the rabbit.²

In working with such compounds, however, it is somewhat difficult to overcome certain theoretical objections which might be raised; for example, many of these compounds are without obvious physiological action in the tissues to which they are distributed. The amounts in question are so small that tests for the excretion of iodine to determine whether or not the compound was broken down and merely the original dye-stuff deposited in the tissue cannot be satisfactorily made. From the chemical point of view, it is difficult to separate completely the iodine compound from the mother dye. There thus remained the possibility that some of the unchanged original dye might be responsible for the vital stain of the tuberculous tissue. It seemed advisable, therefore, to select a typical example for analysis in which the tuberculous tissue in the treated animal could be studied for its iodine content.

We are aware of two previous attempts to study the relation of iodine to tuberculous tissue. The first of these was by Loeb and

¹ Krauss: *Journ. Amer. Chem. Soc.*, xxxvi, p. 961, 1914.

² Lewis: *Arch. of Int. Med.*, x, p. 68, 1912.

Michaud.³ These observers used rabbits. One eye was inoculated with tubercle bacilli and after a suitable interval potassium iodide was administered to the animal. Sometime later the animals were killed and the tuberculous eyes were examined for their iodine content in comparison with the normal eyes. It was found that the tuberculous eyes uniformly showed an appreciably higher iodine content than the normal. In these experiments there would at first sight seem to be a satisfactory control. In other experiments the lymph glands of tuberculous guinea pigs which had been treated with iodoform and ethyl iodide were used. In these cases the presence of iodine at the time of examination was considered by the authors as sufficient evidence that it was derived from the substances administered. Wells and Hedenburg⁴ repeated these experiments on a larger scale. Their methods of analysis were improved. The results were essentially the same as far as the examination of the tissues was concerned.

Our first experiment gave an unexpected result which called attention to a defect in the previous observations. We found in this experiment (see table, p. 315) that in some instances the tuberculous tissue from untreated animals contained appreciable quantities of iodine. This has led us to make some further examination of tuberculous tissue for its iodine content.

Method. The tissues for examination were prepared as follows: Healthy rabbits were chosen and the inoculations were made on one cornea with a virulent culture of tubercle bacilli of bovine type. After an interval of about three weeks, when the lesion was well developed, the animals were killed, the eyes removed, washed free of blood, and the cornea separated from the remainder of the eye. The tuberculous cornea was examined for iodine, the normal cornea being examined as control. As a check on the method of analysis in some instances, all of the remainder of the eyes was analyzed. In the instances where the tuberculous lung tissue was examined in comparison with the normal lung tissue, the animals after a corneal inoculation were allowed to live much longer, until the disease had become generalized. In the early stages of generalization after this method of inoculation, discrete foci of tuberculosis varying from 1 to 5 mm. in diameter are found in the lungs⁵

³ Loeb and Michaud: *Biochem. Zeitschr.*, iii, p. 307, 1907.

⁴ Wells and Hedenburg: *Journ. of Inf. Diseases*, xi, p. 349, 1912.

while the intervening tissue is practically normal. The separation of the tuberculous tissue from the normal lung tissue is relatively easy. In some instances the thyroids of the same rabbits were analyzed for iodine by the same method. The rabbits during the period of the experiment, were confined in separate cages and were fed on the regular diet consisting of a mixture of stale bread and oats soaked for some time in water, with the addition of fresh cabbage two or three times a week.

The method of analysis for iodine was essentially that of Kendall⁵ with certain modifications which were for the most part kindly suggested by Kendall himself. The disintegration of the tissue was carried out in a crucible furnace of our own design which we have recently described⁶ in connection with the analysis of our Trypan-red iodine compounds. Our results are contained in the following table. The results are expressed as milligrams of iodine per gram of dry tissue.

RABBIT NO.	TUBERCULOUS		NORMAL			
	Tissue	Iodine content	Tissue	Iodine content	Tissue	Iodine content
<i>Untreated.</i>						
666*	Cornea	0.276	Cornea	0.157	Rest of eyes	0
671*	Cornea	0	Cornea	0	Rest of eyes	0
683*	Cornea	0	Cornea	0	Rest of eyes	0
992†	Cornea	0	Cornea	0		
997†	Cornea	0.200	Cornea	0	Thyroid	1.383
997†	Cornea	2.67	Cornea	0		
934*	Lung	0	Lung	0	Thyroid	1.565
892*	Lung	0	Lung	0	Thyroid	1.500

Treated with di-iodo-trypan red.

665*	Cornea	1.181	Cornea	0.763	Rest of eyes	0
674*	Cornea	1.107	Cornea	0.679	Rest of eyes	0
673*	Cornea	1.068	Cornea	0.383	Rest of eyes	0
669*	Cornea	1.035	Cornea	0.317	Rest of eyes	0

* Experiment done, May 1913.

† Experiment done, February 1914.

‡ Experiment May 1914.

⁵ Kendall: *Journ. Amer. Chem. Soc.*, xxxiv, p. 894, 1912.

⁶ Krauss: *loc. cit.*

DISCUSSION.

Throughout our experiments it has developed that the tuberculous cornea may contain a very appreciable amount of iodine. The normal cornea has been found to contain iodine but once in the untreated normal animal (Rabbit 666, of table.) In the case of the rabbits treated with our preparation No. 10 (di-iodo-trypan-red), the normal cornea contains much more iodine than the single normal cornea in which iodine was present, and the tuberculous tissue shows still higher values. We feel therefore that by means of our Trypan-red compound we have probably placed iodine in the tuberculous tissue. The fact, however that the tuberculous cornea in the one of the untreated rabbits which showed an iodine content in the cornea gave a much higher value for the tuberculous eye than for the normal eye and the fact that in other experiments iodine has been found in the tuberculous cornea when it was not present in the normal cornea led us to feel that a certain reserve is necessary in drawing conclusions from this form of experimentation. Our experiments of course throw no real light on the reason for the presence of iodine in the untreated tuberculous tissue. They indicate that it is present with some regularity and suggest an interesting field for further study. When one examines the results presented by Loeb and Michaud and by Wells and Hedenburg in the light of our analysis of untreated tuberculous tissue, it becomes apparent that their conclusions also must for the present be accepted with reserve. These authors present no analysis of tuberculous tissue in untreated animals and while some of the figures which they present are high enough to make it probable that the iodine found was derived from the iodine administered, there are a number of other quantities which can hardly be considered significant. Loeb and Michaud give as their highest figure for iodine in the tuberculous eye of rabbits treated with potassium iodide 0.58 mgm. per gram. Our highest figure for the tuberculous tissue is 2.67 mgm. per gram calculated on the dry weight. While it is not specifically stated, their calculation is probably on the basis of the moist weight. Calculated on this basis our high figure for the untreated eye would be 0.53 mgm. per gram or much the same as their high figure for the treated animal. As we know nothing of the influence of various articles

of diet and conditions of the various organs, particularly of the thyroid, on the iodine content of specifically inflamed tissue, it seems impossible at this time to draw final or even valuable conclusions on the basis of any such method of experimentation.

SUMMARY.

Tuberculous tissue derived from animals to which no iodine preparation has been knowingly administered may contain amounts of iodine very appreciably higher than normal control tissue of the same animal. While it is quite probable that tuberculous tissue in animals treated with iodine products may store up iodine, this has been by no means clearly shown in any experiments so far reported. The highest figures for the tuberculous tissue of untreated animals in our experience may equal the highest figures of those reported by others as evidence for the localization in the tissue of iodine intentionally administered.

NOTE ON A CASE OF PENTOSURIA

SECOND COMMUNICATION.

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New York.)

(Received for publication, June 5, 1914.)

In a previous communication¹ we reported some observations on the nature of the pentose in the urine of a person apparently in good health. At the time of the first communication only one derivative of the sugar had been obtained, namely its phenylosazone. Since then the method for obtaining a concentrated solution of the sugar has been improved. This permitted the preparation of the *p*-bromphenylhydrazone. The hydrazone was converted into the free sugar. However, as yet it was not possible to obtain it crystalline. Hence the conclusions regarding the nature of the sugar have to be based on indirect evidence.

At present there exist the following data: first, on the properties of the phenylosazone; second, on the *p*-bromphenylosazone; third, on the optical rotation of the free sugar. The osazone had a melting point of 160 to 163°C., an initial optical rotation of $\alpha = +0.15^\circ$, and equilibrium of $\alpha = +0.57^\circ$ (conditions given in experimental part). The osazone mixed with that of *l*-arabinose melted at 169–178°C., whereas a mixture of equal parts of the urine osazone and of *l*-xylose melted at 203°C.

The *p*-bromphenylhydrazone had a melting point of 130–131°C, and mixed with *l*-xylose-*p*-bromphenylhydrazone, 121–122°C. The rotation of the hydrazone was: initial $\alpha = -0.70^\circ$, equilibrium $\alpha = +1.06^\circ$ (the conditions are given in the experimental part).

The rotation of the sugar was approximately $[\alpha]_D^{20} = +33.1^\circ$.

Discussion of the data. The properties of the osazone permit the conclusion that the urine sugar belongs to the xylose group on the following grounds:

¹ Levene and La Forge: *This Journal*, xv, p. 481, 1913.

First, it was found by Fischer,² and recently pointed out by Zerner and Waltuch,³ that *dl*-xylosazone has a melting point = 205°C., whereas the melting point of *dl*-arabinosazone is only slightly above that of the optically active osazones. Zerner and Waltuch suggested a method for diagnosing a pentose by observing the change in the melting point of its osazone produced by addition of either xylosazone or of arabinosazone of the opposite optical rotation. On the basis of this, a dextrorotatory pentosazone, which shows an elevation of its melting point after being mixed with a levorotatory xylosazone from 163° to 205°C, has to be regarded as xylosazone. The urine pentosazone acted in this manner, and hence its structure is that of a xylosazone.

This conclusion is further substantiated by the character of its mutarotation. It was found in course of this work that the initial optical rotation of xylosazone is lower than the equilibrium rotation, whereas the reverse is true for arabinosazone.

The optical rotation of the urine osazone increases in magnitude on standing. This again is good evidence in support of the xylosazone nature of the urine pentosazone.

A dextrorotatory xylosazone may be derived from one of the following three sugars: from *d*-xylose (according to Fischer's nomenclature), from *l*-lyxose, or from the ketopentose corresponding to these two aldoses. The urine pentose is dextrorotatory hence it cannot be *d*-xylose, for this is levorotatory. It also differs from *d*-xylose by the properties of its *p*-bromphenylhydrazone. The optical rotation of the two substances is markedly different. Their melting points seem to be identical, but the mixed melting point of the two substances shows an unmistakable depression from that of either one.

On the basis of all these considerations the urine pentose cannot be regarded as xylose.

There remain only two other possible explanations of the structure of the urine pentose: It may be regarded either as *l*-lyxose or *l*-ketolyxose (*d*-ketoxylose).

The first assumption is readily removed by a consideration of the properties of the *p*-bromphenylhydrazones. There is a difference

² Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 2486, 1894.

³ Zerner and Waltuch: *Monatsh. f. Chem.*, xxxiv., p. 1639, 1913.

of 30° in their respective melting points. The lyxose derivative is levorotatory, and the range of its mutarotation is smaller than that of the derivative of the urine pentose. Hence also the lyxose possibility has to be abandoned.

By exclusion there remains the last possibility, namely that the urine pentose has the structure of a ketopentose. This assumption in a measure is substantiated by oxidation experiments. By means of nitric acid it was not possible to obtain trioxylglutaric acid. Bromine remained practically without influence on the urine pentose under the same conditions, which brought about a complete oxidation of xylose to xylonic acid. It is noteworthy that fructose is also not attacked by bromine under conditions suitable for oxidation of glycose to gluconic acid.

Hence, all present evidence seems to support the view that the urine pentose analyzed in this work is a ketopentose corresponding to *l*-lyxose or *d*-xylose (Fischer's nomenclature). We expect to continue the work on the products of nitric acid oxidation, and we also expect to prepare the sugar synthetically.

We wish to mention that the sugar on distillation with hydrochloric acid formed very considerable quantities of furfurols, although the yields of this substance were variable.

In conclusion we wish to state that we excluded the possible objection that the ketosugar was produced artificially by an intramolecular rearrangement brought about by the action of lead and barium. A solution of xylose was treated with lead and barium in exactly the same manner as the urine. The lead precipitate of the pentose was freed from lead. It was found that the recovered sugar had the same properties as the original xylose.

EXPERIMENTAL PART.

The urine pentosazone has been prepared directly from the urine⁴ and also from the purified sugar solution. All other osazones used in these experiments were prepared from the pure sugars by heating with phenylhydrazine and acetic acid in the usual manner. *d*-Arabinosazone was obtained from *d*-ribose. All determinations were made by using 0.1 gram substance in 5 cc. pyridine-alcohol mixture with D-light in 0.5 dm. tube.

⁴ This *Journal*, xv, p. 484, 1913.

- d*-Xylosazone. I. Soon after preparation of solution $[\alpha]_D = -0.10$
 After about eighteen hours..... -0.36
 II. The same ten minutes after preparation of solution -0.09
 After one hour..... -0.21
 After twenty-four hours..... -0.43
l-Arabinoxazone. Soon after preparation of solution $[\alpha]_D = +0.55$
 After about eighteen hours..... +0.30
d-Arabinoxazone. Soon after preparation of solution $[\alpha]_D = -0.50$
 After about eighteen hours..... -0.33
Osazone of the urine pentose. I. Soon after preparation of
 solution $[\alpha]_D = +0.15$
 After five hours..... +0.34
 After eight hours..... +0.47
 II. About fifteen minutes after preparation of solution. +0.27
 After eighteen hours..... +0.57

Melting points of osazones.

OSAZONES	CONTRACTED AT °C.	MELTED AT °C.	EFFERVESED AT °C.
<i>l</i> -Arabinose.....	160	166	200
<i>d</i> + <i>l</i> -Arabinose.....	160	170	200
<i>l</i> -Arabinose + <i>d</i> -xylose.....	157	163	180
<i>d</i> -Xylose.....		164	167
Urine pentose.....	155	160	163
<i>l</i> -Xylose + urine pentose.....	194	201	203
Urine pentose + <i>l</i> -arabinose.....	159	169	178

Pentose-p-bromphenylhydrazone.

Fifteen liters of pentose urine were allowed to stand for twelve hours at room temperature with about 75 grams of soy bean meal, during which time a slow stream of carbon dioxide was allowed to bubble through the liquid. Then, without filtering, a warm concentrated solution of barium acetate was added as long as a precipitate continued to be formed. This was filtered off and washed thoroughly with water, the washings being added to the filtrate which was then precipitated with a concentrated solution of lead acetate. The rather large deposit of lead salts was twice washed by triturating in a mortar with water and filtering on a Buchner funnel. The precipitate was then rejected and the combined filtrates which had a volume of about 20 to 25 liters were treated with a solution of basic lead acetate which produces a voluminous white precipitate containing together with other substances prac-

tically all the pentose. At least three washings are necessary to remove sufficiently the adherent solution from the precipitate, after which it was suspended in about 3 liters of water and while being vigorously agitated with a turbine a very rapid stream of carbon dioxide was passed through the suspension. By this treatment the total amount of the pentose together with some other substances are liberated from their metal compounds and pass into solution, while many impurities remain behind in the precipitate. About three-quarters of an hour is sufficient for the operation. The bulky residue must be extracted three times with about 1.5 liters of water to remove all the adhering pentose. The combined filtrates and washings, if their volumes exceeded 5 liters, were concentrated under diminished pressure to that volume, and substances other than pentose present as lead salts transformed into barium salts by addition of hot concentrated barium hydrate solution to distinct alkaline reaction and neutralizing immediately by passing in carbon dioxide. Lead carbonate was removed by filtration. The filtrate was concentrated under diminished pressure at as low a temperature as possible to about 75 cc., and then slowly dropped into 1.5 liters of 100 per cent alcohol, kept agitated with a turbine. One hundred and fifty cc. of dry ether were then added and the voluminous precipitate which settled readily after a few minutes was filtered off with suction. The yield of the pentose was increased somewhat by grinding the barium salts in a mortar with 300 cc. of 100 per cent alcohol, allowing to stand over night, after which the filtrate was combined with the first.

The alcoholic solution of the pentose was concentrated in vacuum to about 45-50 cc. and the precipitation with alcohol and ether repeated. After filtering from the barium salts as before, the filtrate was concentrated in vacuum to about 75-80 cc.,⁵ transferred to a glass dish and the calculated amount of parabromphenylhydrazine added. It was then heated for about ten minutes on the water bath, stirred until the hydrazine was dissolved, allowed to cool and then placed in a vacuum desiccator over solid potassium hydrate. Ordinarily crystallization begins in a few minutes and

⁵ A slight turbidity or even a light precipitate at this stage is of no significance and may be disregarded. It is most convenient to determine the amount of pentose before the solution has been concentrated below 500 cc.

after about twelve hours practically all of the hydrazone has crystallized to a semi-solid cake. It often happened, however, that some was left in the form of a thick light-brown syrup. In either case, addition of 75 to 100 cc. of cold water causes complete crystallization. After about two hours' standing in the refrigerator the hydrazone was filtered off and washed with a little cold water and then without drying extracted with ether as long as colored material was removed. The crude product was recrystallized by dissolving in about 2-2½ parts of alcohol (filtering if necessary) and diluting to about cc. with 4-5 parts of cold water. Soon the hydrazone began to separate in pale yellow plates or scales, which after a time filled the whole volume of the liquid. There is very little loss of material involved in the purification and the yield of the final product which had been washed with ether and dried was about 15 grams, representing about 40 per cent of the total pentose present in the urine.

The hydrazone melted at 128-129° (corr. 130-131°) and decomposed at 154°. The melting point remained unchanged on further recrystallization. A mixed melting point determination with parabromphenylhydrazone of xylose showed a depression of 8° (M.P., 121°; corr. 122°).

0.1265 gram of substance gave 0.1920 gram CO₂ and 0.0538 gram H₂O.

	Calculated for C ₁₁ H ₁₅ N ₃ BrO ₅	Found:
C.....	41.39	41.43
H.....	4.71	4.76

I. 0.3000 gram of substance in 3 cc. of alcohol rotated in 0.5 dm. tube, with D-light:

After five minutes.....	-1.00°
After fifteen minutes.....	-0.93°
After five hours.....	+1.12°
After eighteen hours.....	+1.12°

II. 0.2000 gram of substance in 3 cc. of alcohol rotated in 0.5 dm. tube, with D-light:

After ten minutes.....	-0.70°
After forty minutes.....	-0.55°
After ninety minutes.....	-0.25°
After three hours.....	+0.25°
After five hours.....	+0.72°
After thirty-six hours.....	+0.82°

0.2000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes.....	-0.87°
After two hours.....	-0.86°
After thirty-six hours.....	+1.06°

The barium salts, above referred to, obtained from the alcoholic precipitation have not been thoroughly investigated. They were for the most part crystalline and showed a slight optical activity which might have been due to the presence of some compound of the pentose. They analyzed very close to barium acetate.

	Calculated for $\text{Ba}(\text{CH}_2\text{COO})_2$:	Found:
C.....	18.8	17.9
H.....	2.3	2.9
Ba.....	53.6	45.1

0.2022 gram of substance in 2 cc. water rotated in 0.5 dm. tube with D-light, +0.26°.

Rotation of l-xylose-parabromphenylhydrazine in pyridine and in alcohol.

I. 0.3000 gram of substance in 3 cc. alcohol rotated in 0.5 dm. tube with D-light, +0.06°, and remained unchanged for forty-eight hours.

II. 0.3000 gram of substance in 3 cc. alcohol + a few drops of pyridine rotated in 0.5 dm. tube with D-light, -0.06°; after eighteen hours, +0.06°.

III. 0.3000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes.....	-1.22°
After eighteen hours.....	-1.00°
After forty-eight hours.....	-0.42°

Parabrom- and paranitrophenylhydrazones of d-lyxose.

Both of these derivatives possess better properties than the benzylphenylhydrazone described by Ruff.⁶ They were prepared by bringing together the calculated amounts of d-lyxose and the respective hydrazines in 75 per cent alcoholic solution. Both were recrystallized from 95 per cent alcohol.

d-Lyxose-p-bromphenylhydrazone melted at 158° (corr. 161.5°).

0.1530 gram of substance gave 0.2343 gram CO_2 and 0.0688 gram H_2O .

	Calculated for $\text{C}_{11}\text{H}_{15}\text{N}_2\text{BrO}_4$:	Found:
C.....	41.39	41.38
H.....	4.71	4.99

⁶ Ruff: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 552, 1899; Ruff and Ollendorff: *ibid.*, xxxiii, p. 1798, 1900.

0.2000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes.....	+1.06°
After twenty-four hours.....	+0.26°
After forty-eight hours.....	+0.26°

d-Lyxose-*p*-nitrophenylhydrazone melted at 169°C. (corr. 172°).

0.1547 gram of substance gave 0.2655 gram CO₂ and 0.0768 gram H₂O.

	Calculated for C ₁₁ H ₁₃ N ₃ O ₆ :	Found:
C.....	46.36	46.27
H.....	5.26	5.51

p-Bromphenylhydrazones of pentoses.

PENTOSE	M. P. °C.	SOLVENT	CONCENTRATION	[α] _D IN 0.5 DM. TUBE	
				Initial	Final
<i>d</i> -Lyxose.....	158	Pyridine	1 gram in 10 cc.	+1.41	+0.34
<i>l</i> -Xylose.....	128-129	Alcohol	1 gram in 10 cc.	+0.06	+0.06
<i>l</i> -Xylose.....		Pyridine	1 gram in 10 cc.	-1.22	-0.42
Urine pentose....	128-129	Alcohol	1 gram in 10 cc.	-1.00	+1.12
Urine pentose....		Pyridine	1 gram in 10 cc.	-0.87	+1.06

Cleavage of the urine pentose-parabromphenylhydrazone with benzaldehyde.

2.20 grams hydrazone dissolved in about 75 cc. of hot water were heated three quarters of an hour on the water bath with 0.8 gram of benzaldehyde. The hydrazone of benzaldehyde begins to separate at once and at the end of the experiment, after having been filtered off, washed and dried, weighed 1.83 grams, which is 97 per cent of the calculated 1.89 grams. In the filtrate were present by calculation 1.035 grams of pentose. The solution was extracted four times with ether and then concentrated in vacuum to 40 cc., placed in a measuring flask and the volume made up to 50 cc.

This solution showed a rotation in 2 dm. tube at 25° with D-light of +1.38°.

$$[\alpha]_D^{25} = +33.15^\circ$$

⁷ This value must be regarded as a minimum.

Reduction of Fehling solution.

I. 2 cc. of solution, containing 0.0412 gram pentose as calculated from the amount of hydrazone, corresponded to 11.3 cc. $\frac{N}{10}$ NH_4CNS (0.0717 gram Cu).

II. 2 cc. of solution gave 11.2 cc. $\frac{N}{10}$ NH_4CNS (0.0710 gram Cu).

Factor = 0.577.

Attempts to oxidize the pentose with bromine.

Two grams of pentose, calculated from reduction with Fehling solution (the reduced copper being estimated by the Volhard method) in 90 cc. of water were allowed to stand for two days at 25° with an excess of bromine. After this time, 2 cc. were boiled to remove the bromine and found by reduction to correspond to 11 cc. $\frac{N}{10}$ sulphocyanate solution = 0.0697 gram Cu, or using the above-mentioned factor, 0.0398 gram pentose in 2 cc. or 0.179 gram in 90 cc. (85 per cent of the original amount).

A parallel experiment made with 2 grams of xylose under the same conditions showed only 0.04 gram pentose in the whole solution (12 cc. corresponded to 1.5 cc. $\frac{N}{10}$ sulphocyanate solution).

Attempts to oxidize with nitric acid in the usual manner the sugar syrup obtained from the parabromphenylhydrazone were unsuccessful. Nothing could be isolated from the reaction product.

The relation between the reducing power of d-xylose to rotation after precipitation with basic lead acetate.

Four grams of *d*-xylose dissolved in 1 liter of water were precipitated with basic lead acetate and barium hydrate. The precipitate was decomposed with sulphuric acid, the excessive sulphuric acid removed with lead carbonate, the excess of the latter with hydrogen sulphide, and the solution concentrated to about 75 cc.

1 cc. corresponded to 8 cc. of $\frac{N}{10}$ sulphocyanate solution (Volhard).

1 cc. corresponds to 0.0312 gram of xylose; α in 2 dm.-tube and D-light = 1.11.

$$[\alpha]_D = -18.16^\circ$$

COMPARISON OF THE CARBON-DIOXIDE OUTPUT OF NERVE FIBERS AND GANGLIA IN LIMULUS.

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(Received for publication, May 11, 1914.)

Previous studies have shown that pieces of living nerve trunks which contain no nerve cells give off carbon dioxide at a rapid rate, and that the rate varies with the state of irritability of the fiber.¹ This paper contains a record of the carbon-dioxide output of nerve ganglia. It seemed of interest to determine whether a nerve tissue containing ganglionic cells produces more or less carbon dioxide per gram of substance than the nerve fibers.

The ganglion chosen was the dorso-median cord in the heart of the king-crab (*Limulus polyphemus*). This is an elongated, automatic ganglion, which has been shown by Carlson² to be the direct cause of the heart beat. It was isolated in the manner described by him, the operation taking but a few moments, since no precaution against injuring the heart muscle was necessary. The main dorsal cord, separated from the muscle by the ectocordium, was lifted at the posterior end, in the region of the last pair of ostia, and the entire length taken, up to the region of the first segment. The greyish white color of the cord makes it readily distinguishable in the living specimen from the adjoining connective tissue.

The cord thus isolated was quickly weighed to a milligram, and its respiration at once determined by Tashiro's apparatus. Both the biometer³ and apparatus III⁴ were used. The volume of the respiration chamber was in every case 15 cc.

¹ Tashiro: *Amer. Journ. of Physiol.*, xxxii, pp. 107-136, 1913; also Tashiro and Adams: *ibid.*, xxxiii, p. xxxviii, 1914.

² Carlson: *ibid.*, xii, pp. 67-74, 1904.

³ Tashiro: *ibid.*, xxxii, p. 141, 1913.

⁴ Tashiro: *This Journal*, xvi, p. 485, 1914.

330 Carbon-dioxide Output of Nerve Tissue

The animals were brought daily from the "live car," where they had been kept without feeding since May.⁵ They were to all appearances healthy.

As is shown in Table I, a centigram of ganglionated cord gives off in ten minutes, at 22.8–23°C. from 2.3×10^{-7} to 4.7×10^{-7}

TABLE I.

Carbon-dioxide production in ganglia in Limulus heart.

	DATE	TEMPERATURE OF ROOM	SEX OF ANIMAL	NO. OF CC. WHICH GIVES NO PPT. CALCULATED FOR 10 MG.—10 MIN.	NO. OF CC. WHICH GIVES PPT., CALCULATED FOR 10 MG.—10 MIN.
A.....	Aug. 31	23.2°	♂	3.41	6.29
	Aug. 31	23.0	♂	2.04	3.06
	Aug. 31	23.0	♂	2.04	
	Aug. 31	22.8	♂		4.12
	Sept. 1	23.5	♂	1.98	2.97
	Sept. 1	23.5	♂	1.98	
	Sept. 1	23.5	♂	2.97	3.96
	Sept. 1	23.5	♂	1.98	
	Sept. 1	23.5	♂	2.97	3.96
B.....	Aug. 31	23.0	♀	2.58	
	Aug. 31	23.0	♀	3.87	
	Aug. 31	23.0	♀	5.16	6.45
	Aug. 31	22.9	♀	4.60	8.87
	Sept. 1	23.2	♀	2.60	8.59

A. Since in one case 3.41 cc. for 10 mg. -10 min. failed to give a precipitate, and in another case as little as 2.97 cc. gave a positive test, the minimum volume in which is contained the minimum CO₂ must lie between these figures. Taking an average, therefore, we get 3.19 cc. Then, $1 \times 10^{-7} \times \frac{15}{3.19} = 4.7 \times 10^{-7}$ gram CO₂ at 23°-23.5°

B. $1 \times 10^{-7} \times \frac{15}{6.45} = 2.3 \times 10^{-7}$ gram CO₂ at 23° maximum error - 0.2×10^{-7} gram.

gram of CO₂, the rate being less in the larger individuals, which were females. This amount of carbon dioxide is very small when compared with the output, per centigram for ten minutes, of the claw nerve of the spider-crab (Table II), which without stimulation

⁵ It is generally believed that they do not feed after they are caught.

TABLE II.

Summary of carbon-dioxide production from various nerve tissues.

ANIMAL	SEX	NERVE	TEMPERATURE	GM. CO ₂ GIVEN OFF CALCULATED FOR 10 MGM.—10 MIN.	ESTIMATED BY
			°C.		
Limulus poly- phemus	♂	Nerve cord of heart (30-34 mgm.)	23-23.5	4.7×10^{-7}	Tashiro, Adams
	♂	Nerve cord of heart (51 mgm.)	23	2.4×10^{-7} (-0.4×10^{-7})*	Tashiro, Adams
	♂	Nerve cord of heart (52 mgm.)	23	2.3×10^{-7} (-0.2×10^{-7})*	Tashiro, Adams
	♂	Claw nerve	23	2.6×10^{-7}	Tashiro, Adams
	♀	Optic nerve, whole	17.8	2.6×10^{-7}	Tashiro†
	♀	Optic nerve, proximal part	22.5	3.0×10^{-7}	Tashiro†
	♀	Optic nerve, distal part	22	5.0×10^{-7}	Tashiro†
Labinia canali- culata		Claw nerve, whole	15-16	6.7×10^{-7}	Tashiro†
		Claw nerve, whole	20.2	7.9×10^{-7}	Tashiro, Adams‡
		Claw nerve, proximal part	21.4	8.0×10^{-7} (-)*	Tashiro†
		Claw nerve, distal part	23.2	3.7×10^{-7} (-1.0×10^{-7})*	Tashiro†
		Claw nerve, whole, when stimulated	14-16	16.0×10^{-7}	Tashiro†
Rana pipiens		Sciatic, resting	19-20	5.5×10^{-7}	Tashiro†
		Sciatic, stimulated	20-22	14.2×10^{-7}	Tashiro†

* Maximum possible error.

† Quoted from *CO₂-Production Gradient*, now in press.‡ Quoted from *Amer. Journ. of Physiol.*, xxxii, p. 127, 1913.§ Quoted from *Effect of Anaesthetics on CO₂-Production of Nerve Fibre*, now in press.

gives off from $6 - 8 \times 10^{-7}$ gram, and when stimulated, 16×10^{-7} gram. If, however, the comparison be made with the claw nerve or optic nerve of *Limulus*, the rate in the ganglion is found to be about the same as that in the fiber. The claw nerve gives only about 2.6×10^{-7} gram of CO_2 . The optic nerve gives somewhat more, *i.e.*, $2.6 - 5 \times 10^{-7}$ gram, depending on whether the proximal (nearer to brain) or the distal (nearer to retina) portion is taken.

It appeared from the determinations that the heart ganglion gives off about the same amount of carbon dioxide per gram of substance as the nerve fiber. Certainly there is no marked superiority of carbon-dioxide output by the ganglion; if anything its rate is a little lower. This result may be due to an equality of metabolism in the two cases, or the injury may have raised the rate more in one case than in the other. It is also possible that in the ganglion the amount of non-nervous tissue was somewhat greater than in the nerve trunk.

Another interesting result was to show that the rate of metabolism (carbon-dioxide output) in both fibers and ganglia of *Limulus* is decidedly lower than in the nerves of the spider-crab (*Libinia canaliculata*) or the frog (*Rana pipiens*) (see Table II). This may be correlated with the very sluggish behavior of *Limulus*, and its power of living for very long periods without food, and with very little air.

In this connection it is interesting to note the connection between the rate of the nerve impulse and its carbon-dioxide production. The rate of the impulse, determined by Carlson,⁶ for the ambulacral nerve of *Limulus* is 3.25 mm., while for the corresponding nerve of the spider-crab it is 6 mm. As shown in Table II, this ratio is very nearly the same as that found for the carbon-dioxide output. Our results suggest strongly that the low rate of conduction of the nerve impulse is due to, or correlated with, the low rate of metabolism (carbon-dioxide output).⁷

⁶ Carlson: *Amer. Journ. of Physiol.*, xv, p. 136, 1906.

⁷ It must be understood here that we do not mean to say that any nerve which gives more CO_2 , gram for gram, has a higher velocity of nerve impulse. Comparison should be made between corresponding nerves of different animals, or different nerves of the same animal. (See Carlson: *loc. cit.*)

In the course of the work it was found that the carbon-dioxide output per centigram of nerve cord was generally greater in the males than in the females. In the males the output was on the average about 4.7×10^{-7} gram, and in the females, 2.3×10^{-7} gram. (Table I.) The male *Limulus* is usually considerably smaller than the female. If a male and a female of approximately the same size are compared, the difference largely disappears, but whether this difference is due to size or age or to sex is difficult to determine. It is probable in this case that the smaller female is younger, and has on that account a higher metabolism. We hope to make further observations regarding the importance of these various factors, since the results so far obtained, which are presented in Tables III and IV, are too few to enable us to form any very definite opinion.

TABLE III.

Comparison of male and female of approximately the same size.

	DATE	TEMPERATURE	SEX AND WEIGHT OF CORD	NO. OF CC. WHICH GIVES NO PPT., CALCULATED FOR 10 MG.—10 MIN.	NO. OF CC. WHICH GIVES PPT., CALCULATED FOR 10 MG.—10 MIN.
A.....	Sept. 1	23.2°	♂ 51 mg.	1.53	
	Sept. 1		Same ♂	3.06	
	Sept. 1		Same ♂	4.59	6.12
	Sept. 1		Same ♂	3.06	6.12
	Sept. 1		Same ♂	3.06	6.12
	Sept. 1	23.0	♂ 51 mg.	2.55	6.42
B.....	Sept. 1	23.2	♀ 52 mg.	2.60	8.59
	Aug. 31	23.0	♀ 43 mg.	2.58	
	Sept. 1		Same ♀	3.87	
	Sept. 1		Same ♀	5.16	6.45
	Sept. 1	22.9	♀ 46 mg.	4.60	

A. 1.0×10^{-7} gram $\times \frac{15}{6.12} = 2.4 \times 10^{-7}$ gram ($- 0.4 \times 10^{-7}$ gram).
(See Table II.)

B. 1.0×10^{-7} gram $\times \frac{15}{6.45} = 2.3 \times 10^{-7}$ gram ($- 0.2 \times 10^{-7}$ gram).
(See Table II.)

TABLE IV.

Comparison of different sizes of male (in different age.)

	DATE	TEMPERATURE OF ROOM	SEX OF ANIMAL	WEIGHT OF CORD	NO. OF CC. WHICH GIVES NO PPT., CALCULATED FOR 10 MG.—10 MIN.	NO. OF CC. WHICH GIVES PPT., CAL. FOR 10 MG.—10 MIN.
A.....	Aug. 31	23.2°	♂	31 mg.	3.41	6.29
	Aug. 31	23.0	♂	34 mg.	2.04	3.06
	Aug. 31	23.0	Same ♂	34 mg.	2.04	
	Aug. 31	22.8	♂	37.5 mg.		4.12
	Sept. 1	23.5	♂	30 mg.	1.98	2.97
	Sept. 1	23.5	Same ♂	30 mg.	1.98	
	Sept. 1	23.5	Same ♂	30 mg.	2.97	3.96
	Sept. 1	23.5	Same ♂	30 mg.	1.98	
	Sept. 1	23.5	Same ♂	30 mg.	2.97	3.96
B.....	Sept. 1	23.2	♂	51 mg.	1.53	
	Sept. 1	23.2	Same ♂	51 mg.	3.06	
	Sept. 1	23.2	Same ♂	51 mg.	4.59	6.12
	Sept. 1	23.2	Same ♂	51 mg.	3.06	6.12
	Sept. 1	23.2	Same ♂	51 mg.	3.06	6.12
	Sept. 1	23.0	♂	51 mg.	2.55	6.42

A. 4.7×10^{-7} gram CO_2 .B. 2.4×10^{-7} gram CO_2 (-0.4×10^{-7} gram.) (See Table II.)

CONTRIBUTIONS TO THE BIOCHEMISTRY OF IODINE.

I. THE DISTRIBUTION OF IODINE IN PLANT AND ANIMAL TISSUES.

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INTRODUCTION.

From a biological standpoint the two facts of paramount importance in the history of iodine are the discovery of the element itself in kelp in 1811 by Courtois, and the discovery of its presence in the thyroid gland by Baumann in 1895. Subsequent to this date investigations have been directed chiefly with the aim of discovering the function, if any, of iodine, in the thyroid. A very large number of papers have appeared, whose chief result seems to be to throw into doubt all the earlier conclusions derived from the work of Baumann and his followers (as for example that the iodine of the thyroid is present organically combined in a simple compound "iodothylin," to which is due the activity of the gland as a secretory organ) without presenting a more defensible theory.¹

Various conflicting statements have been put forward as to the presence or absence of iodine in other mammalian tissues, especially those of the glands of internal secretion. More definite data are available for certain invertebrates. Thus the presence of iodine in sponges was discovered by Fyfe in 1819,² and confirmed by numerous investigators,³ while Drechsel first pointed out its presence in

¹ See in this connection Swale Vincent: *Internal Secretion and the Ductless Glands*, Arnold, London, 1912, p. 311; Biedl: *Innere Sekretion*, 2te Aufl. 1913, i, p. 211.

² Fyfe: *Edin. Phil. Journ.*, i, p. 254, 1819.

³ Cf. Harnach: *Münch. med. Wochenschr.*, xliii, p. 196, 1896.

corals.⁴ Numerous investigations have shown that it is present in almost all sea-weeds.

The fact that when a diet rich in iodine is administered to an animal such as the dog, the iodine content of the thyroid is markedly and rapidly increased, was pointed out by Baumann himself,⁵ and has been repeatedly confirmed. The known variations of iodine content of the gland in different species of animals (as distinct from the marked individual variations which occur in the same species) were attributed to this cause by Róos,⁶ and the theory is usually accepted, that the diet of an animal is the determining factor of the amount of iodine in its thyroid. Reasoning from this, I was led to examine the thyroids of certain elasmobranch fishes, since these, living in a medium containing iodine in distinct amount, should show high figures for the iodine content of the thyroid if the theory were correct. I found⁷ that iodine was present in marked amount, and, for the female dog-fish *Scyllium canicula*, obtained a value higher than any previously recorded. These results suggested the desirability of examining the various forms of sea-life in greater detail. The examination of the iodine compounds in sponges and corals, leading to the definite identification of 3, 5-di-iodo-1-tyrosine as one of the forms in which iodine is linked in organic tissue,⁸ and not improbably in the thyroid itself,⁹ indicated that further light might be thrown on the problem of the form or forms in which iodine is held in living tissues, by an investigation over a wider field.

With the permission of the Biological Board of Canada I was able to collect material at their Pacific Coast Station at Nanaimo, B. C., during August, 1913. I obtained a large number of specimens of different species of Algae, and specimens of representative species of most of the animal phyla. The selection of the latter was made more or less at random, and analysis of the different tissues examined was also not systematic; the investigation undertaken

⁴ Drechsel: *Zeitschr. f. Biol.*, xxxiii, p. 85, 1896.

⁵ Cf. Baumann and Goldmann: *Münch. med. Wochenschr.*, xliii, p. 1153, 1896.

⁶ Roos: *Zeitschr. f. physiol. Chem.*, xxviii, p. 40, 1899.

⁷ Cameron: *Biochem. Journ.*, vii, p. 466, 1913.

⁸ See for example, Wheeler and Mendel: this *Journal*, vii, p. 1, 1909.

⁹ Cf. Oswald: *Arch. f. exp. Path. u. Pharm.*, lx, p. 115, 1908; Nürnberg: *Biochem. Zeitschr.*, xvi, p. 87, 1909.

was rather preliminary, with the purpose of indicating the direction for further work. Complete examination of the tissues of the dog-fish, *Squalus sucklii*, was carried out. I have employed Hunter's method of iodine analysis¹⁰ throughout. Since most of the previous results have been obtained by the use of Baumann's method of analysis,¹¹ or of some modification of it, and since (see next section) there is considerable evidence that this method frequently yields too low values, and does not detect small amounts, it seemed desirable to examine ordinary mammalian tissue completely by the newer method. I have done this for the dog and the rabbit.

It seemed desirable also in this paper to review the large amount of data scattered over the literature of the past thirty years, with a view to determine exactly our present knowledge of iodine in different tissues, and what gaps in this knowledge require attention. In the section dealing with my actual results I therefore include a brief resumé of such previous data as I have been able to examine, bearing on the presence of iodine in normal tissue.

In this communication I shall consider normal tissue only, and shall not deal with the function of the thyroid itself, nor with the function (if any) of iodine in living tissue, nor with the iodine content of tissues where iodine has been administered experimentally or therapeutically to the organisms.

METHODS OF ANALYSIS OF IODINE.

Three methods of analysis of iodine in organic tissue have been employed by different investigators, that of Baumann (or some modification of it), that of Hunter, and that of Bourcet. I propose to outline these briefly, to consider their accuracy and degree of sensibility, and to deduce from these what relative stress can be laid on results obtained by each method.

Baumann's method¹² consists of a modification of that originally proposed by Rabourdin.¹³ The organic material to be analyzed (one or two

¹⁰ Hunter: this *Journal*, vii, p. 321, 1910.

¹¹ See Baumann and Roos: *Zeitschr. f. physiol. Chem.*, xxi, p. 489, 1895.

¹² Baumann and Roos: *loc. cit.*

¹³ Rabourdin: *Ann. d. Chem.*, lxxvi, p. 375, 1850.

grams) is fused with sodium hydroxide, and the charred mass oxidized with potassium nitrate. The fused mass is dissolved in water and acidified, and the iodine liberated in the presence of chloroform (Baumann), carbon disulphide (Oswald¹⁴), or carbon tetrachloride (Riggs¹⁵). The iodine solution so obtained is determined by comparison with a standard solution of known strength in the same solvent (Baumann), by comparison with a standard solution of Fuchsin S (Seidell¹⁶), or by titration against thiosulphate (Tambach¹⁷), or potassium permanganate (Bray and Mackay¹⁸).

Baumann claimed that his method permitted the detection of 0.076 mgm. of iodine. As modified by later workers, it is said to be more delicate. Seidell, who has employed it extensively, considers that 0.01 mgm. per gram can be detected, but that with larger quantities of iodine variations of the order of 0.05 mgm. are to be expected.

Hunter's method¹⁹ consists in fusion of the dried material (usually 1 gram) with a mixture of sodium carbonate, potassium carbonate, and potassium nitrate, dissolution in water, oxidation of iodide to iodate by addition of hypochlorite and subsequent acidification with phosphoric acid, removal of excess chlorine by boiling, subsequent addition of potassium iodide in excess to the cooled liquid (giving six times the original amount of iodine), and titration of the iodine against dilute thiosulphate, using starch as indicator. This method solves the difficulty of the production of iodate in the initial fusion, which, as pointed out by Riggs,²⁰ may result in considerable loss of iodine in the Baumann method.

Hunter has measured the limits of accuracy of his method by direct addition of iodine to acidified solutions of the fusion mixture, and titration with thiosulphate. Starch gives a perceptible color when 0.03 mgm. of iodine is present, corresponding therefore to 0.005 mgm. in the original tissue. He considers²¹ that since blank tests give occasionally slight positive results, the limit of detectability should be taken as twice this amount, and that quantities less than 0.01 mgm. should not be reported. Duplicate analyses of material containing from 1 to 2 mgm. of iodine should not differ by more than 0.03 mgm.

A very careful comparison of the two methods has been carried out by Seidell,²² who confirms the accuracy of Hunter's method, and finds that Baumann's method usually yields lower results, from which it may

¹⁴ Oswald: *Zeitschr. f. physiol. Chem.*, xxiii, p. 265, 1897.

¹⁵ Riggs: this *Journal*, vi, *Proc. Soc. Biol. Chem.*, p. xli, 1909.

¹⁶ Seidell: *ibid.*, iii, p. 391, 1907.

¹⁷ Tambach: *f. Biol.*, xxxvi, p. 549, 1898.

¹⁸ Bray and Mackay: *Journ. Amer. Chem. Soc.*, xxxii, p. 1139, 1910.

¹⁹ Hunter: *loc. cit.*

²⁰ Riggs: *Journ. Amer. Chem. Soc.*, xxxi, p. 711, 1909.

²¹ Hunter: *loc. cit.*, p. 348.

²² Seidell: this *Journal*, x, p. 95, 1911.

be concluded that minute quantities of iodine (of the order, say, of 0.05 mgm.) may frequently escape detection if Baumann's method is employed. Hunter's method, however, as Kendall has pointed out,²³ sometimes leads to errors when only very small quantities of iodine are present, through the formation of minute traces of oxychlorine acids, which subsequently react with the added iodide, liberating iodine. To remedy this defect Kendall has proposed a modification in which bromine is used to oxidize the iodide, after an initial fusion with sodium hydroxide and a small quantity of potassium nitrate; he claims to get uniformly correct results with it. So far, however, no data has been published, based on the employment of this method, so that it need not be considered further here.

Bourcet's method²⁴ appears to be the most accurate which has so far been devised. It consists essentially of fusion of large amounts of substance with potassium hydroxide in a nickel crucible, extraction with boiling water, removal of potassium hydroxide from the extract by repeated additions of alcohol, and subsequent concentrations, evaporation to dryness, partial ignition, redissolution in water, and estimation of the iodine by Roubourdin's method (addition of nitrite in presence of acid and carbon disulphide, and colorimetric comparison). Bourcet claimed that he could estimate 0.03 mgm. iodine per 100 grams of material by this method, without loss, so that the limit of detection must be placed considerably below this figure.

Results from the use of Bourcet's method are to be considered as certain indications of the presence of iodine, but the method of initial fusion suggests that loss may occur, as in Baumann's method, through the formation of iodate, so that all figures based on this method are to be regarded as minimal while minute quantities with small amounts of material may escape detection.

Justus, having devised a histochemical method by which he claimed that he was able to show that every cell nucleus contained iodine,²⁵ and since Bourcet has obtained negative results for the brain, eye, and pancreas, was led to devise a simpler method,²⁶ somewhat similar to that of Bourcet, but much less exact, and obtained therefrom positive results with all tissues, in conformance with his theory. His histochemical method has been tested by Babij,²⁷ and found incorrect. His results obtained by his chemical method are probably all within the limit of error of his method, as has been pointed out by Zoeppritz.²⁸

Blum and Grützner²⁹ have recently suggested a method somewhat simi-

²³ Kendall: *Journ. Amer. Chem. Soc.*, xxxiv, p. 904, 1912.

²⁴ Bourcet: *Compt. rend. de l'Acad. des Sci.*, cxxviii, p. 1120, 1899.

²⁵ Justus: *Arch. f. path. Anat. u. Physiol.*, clxx, p. 501, 1902.

²⁶ Justus: *ibid.*, clxxvi, p. 1, 1904.

²⁷ Babij: *Ber. d. bot. Gesellsch.*, xxxi, p. 35, 1913.

²⁸ Zoeppritz: *Münch. med. Wochenschr.*, lix, p. 1898, 1912.

²⁹ Blum and Grützner: *Zeitschr. f. physiol. Chem.*, lxxxv, p. 429, 1913.

lar to that of Hunter, in which barium peroxide is used as the oxidizing agent. So far no results for organic tissues have been put forward by these authors.

I have used Hunter's method to obtain the results contained in this paper. I have almost always employed 0.5 gram of dried material, where sufficient material was available, together with 11 grams of the combustion mixture. When the solution was only slightly acid, and was boiled for from one to one and one-half hours, I usually obtained accurate results, blank tests yielding no trace of iodine. Not seldom, however, slight traces of iodine were obtained in such blank tests (I attribute these to the cause suggested by Kendall), and these necessitated frequent repetitions of all results in which only minute traces of iodine were found. I have indicated below those results, where from insufficiency of material, I was unable to obtain satisfactory confirmation, by placing the figures in brackets. I have accepted negative results as correct.

I have carried out some experiments to test the minimal quantity of iodine detectable by the method as I have employed it. I added known quantities of dilute potassium biniodate to 150 cc. of water (the amount of solution I obtained for titration was usually of this order), then excess of potassium iodide and starch, as in the actual titration. I found that 0.006 mgm. biniodate gave a perceptible coloration, while 0.004 mgm. gave no coloration. The corresponding amounts of iodine are 0.004 and 0.0025 mgm., so that this result is in good agreement with that of Hunter already quoted. It indicates that when 0.5 gram of material is taken, iodine is detectable when 0.0008 per cent is present, and is not detectable when 0.0005 per cent is present. I have tested the correctness of this result by adding known amounts of a very dilute solution of potassium iodide (0.0004 per cent) to 0.5 gram of fibrin, and carrying out analyses in the usual way. The following results were obtained:

<i>Amount of potassium iodide taken.</i>	<i>Amount of iodine taken.</i>	<i>Amount of iodine found per cent</i>
0.002 mgm. = 0.0003 per cent of 0.5 gram.....		0
0.002 mgm.		0
0.004 mgm. = 0.0006 per cent of 0.5 gram.....		0.0007
0.004 mgm.		0.0019
0.006 mgm. = 0.0010 per cent of 0.5 gram.....		0.0011

These results substantially confirm Hunter's limits. The fourth result indicates that error due to oxychlorine acids already referred to. I conclude in agreement with Hunter that the method allows the detection of 0.001 per cent (Hunter's calculations are based on the starch end point, and do not therefore refer to the original amount of substance taken), while negative results indicate that less than 0.001 per cent is present. I have tested the accuracy of the method for larger amounts of iodine by numerous controls and duplicate analyses, with concordant results. (Where less than 0.5 gram of material was employed, a negative result had, of course, a correspondingly less significance.)

Summing up the comparison of the three chief methods I have outlined above, Baumann's method shows the presence of not less than from 0.01 to 0.08 mgm. iodine, according to the modification employed, and the investigator employing it. This corresponds to between 0.001 and 0.008 per cent when 1 gram of material is taken. For reasons stated above, however, quantities of the higher order are probably frequently not detected when the method is employed. Hunter's method permits the detection of 0.001 per cent of 0.5 gram material, and the error is of the order of 1 per cent when quantities of iodine of 1 mgm. or more are present.³⁰

Bourcet's method permits the measurement of 0.00003 per cent of iodine with accuracy, so that amounts much less than this are probably detectable.

Results based on Hunter's method are approximately correct, while those based on Baumann's or Bourcet's are minimal. Hunter's method is a convenient one for the rapid estimation of iodine, where it is likely to occur in moderate amount.

In the tables in the following section I have indicated the method employed by the abbreviations: H (Hunter); Bn (Baumann); and Bt (Bourcet).

THE DISTRIBUTION OF IODINE IN PLANT AND ANIMAL TISSUES.

In this section it will be most convenient to deal systematically with the different species for which results have been obtained throughout the plant and animal kingdoms, giving first my own results, and then summarizing them in tables with other published data. Before proceeding to do this however, it will be convenient to indicate very shortly the present state of our knowledge regarding the distribution of iodine in inorganic matter.

Abel and Halla (Abegg's "Handbuch der anorganischen Chemie, Bd. IV, ii Abt., 1913, S. 340) makes the following statement as to the general distribution of iodine: "Jod ist in der Natur allgemein verbreitet, tritt jedoch stets nur in sehr geringen Mengen auf. Chatin hat zuerst die neuerdings bestätigte Lehre vom allgemeinen Vorkommen des Jods aufgestellt. Ueber den durchschnittlichen Prozentgehalt der gesamten Erdrinde an Jod liegen voneinander stark abweichende Angaben vor. J. A. L. Vogt nimmt 1×10^{-4} Proz., Ackroyd dagegen 5.8×10^{-3} Proz. an.

³⁰ Hunter: *loc. cit.*, p. 348.

Die feste Erdkruste enthält nach Vogt rund 10^{-8} Proz. Im Meerwasser sollen ungefähr 10^{-3} Proz. vorhanden sein; Gautier fand einen etwa 5mal kleineren Gehalt. Bezüglich seines Beitrages zum Aufbau der Erdrinde steht Jod unter den übrigen Elementen an 28. Stelle. Jod scheint, nach diesen geringen Mengen zu urteilen, keinen wesentlich chemisch-geologischen Einfluss ausgeübt zu haben. Das Verhältniss von Br : J ist im Meerwasser und den Gesteinen annähernd gleich und beträgt 1 : 10 bis 12, das von Cl : J = 1 : 0,00012."

According to the accounts given by these authors, iodine occurs chiefly in the form of iodides. In the Chili and Bolivian saltpetre beds iodates and periodates are found. The sea is the great store-house of iodine, which is also found frequently in mineral springs.³¹

It would therefore appear that iodine is of such wide distribution that all living tissue having a selective "affinity" for the element should be able to store it in distinct quantity, while marine organisms possess special facilities for obtaining relatively large quantities.

As mentioned above, Chatin was the first to state the wide distribution of iodine. He claimed to find it in small amounts in air, rain-water, soils, and almost all food material, etc.³² It was shown conclusively at a later date that his results and those of his pupils were spoiled through the use of chemicals containing iodine,³³ so that no reliance can be placed on his figures, although later, more accurate work has confirmed his conclusions.

I have been unable to find any accurate recent data for the iodine content of sea-water, and no data bearing on the variation of this with changing salinity and specific gravity. The limits of the values found by different authors have been quoted above. The variations may in part be due to different salinity (greater or less contamination with fresh water, according to the source of the sea-water examined). The most recent work on sea-water appears to be that of Gautier.³⁴ He found that sea-water, obtained in the English Channel, 40 kilometers from the coast, contained no inorganic iodine, 1.8 mgm. organic iodine, and 0.52 mgm. iodine in

³¹ Cf. also the account given in Gmelin's *Handbuch der anorganischen Chemie*, Bd. I, Abt. ii, p. 287, 1909.

³² Chatin: *Compt. rend. de l'Acad. des Sci.*, xxxiii, pp. 519, 529, 584, 1851.

³³ Cf. for example, Macadam: *Edinburgh Phil. Journ.*, liii, p. 315; *Chem. Soc. Quarterly Journ.*, vi, p. 166, 1854; de Luca: *Journ. de pharm.*, (iii), xxxii, p. 414; xxxiii, p. 32; Nadler: *Journ. f. prakt. Chem.*, xcix, p. 83. It is interesting to note that Macadam after finding that iodine was present in neither air, rain-water, nor snow, was able to show its general distribution, and its presence in a number of plants. From its presence in edible plants especially, he concluded that it should be present in the higher mammals, including man, although he was unable to demonstrate this.

³⁴ Gautier: *Compt. rend. de l'Acad. des Sci.*, cxxviii, p. 1069; cxxix, p. 9, 1899.

different organisms (removed by filtration), per liter. The organisms, examined microscopically, appeared to consist of ligneous fibres, sponge spicules, minute fragments of insects, flagellates, rotiferas, and especially of diatoms. Water from the surface of the Mediterranean (Gulf of Lyons, 11 kilometers from the coast) contained no inorganic iodine, 1.960 mgm. organic iodine, and 0.286 mgm. present in organisms, per liter. Gautier considered that since water at 780 meters contains no living Algae, inorganic iodine should be present in greater amount, the greater the depth, and he found, concordantly, that at 880 meters depth (Gulf of Lyons) the water contained per liter, inorganic iodine, 0.150 mgm., organic iodine, 2.130 mgm., iodine in organized matter, 0.100 mgm. The corresponding figures at 980 meters depth were 0.305, 1.890, and 0.065.

Some data have been published from which it would appear that iodine may also occur in certain fresh waters, chiefly or solely in organic combination. According to Montanari³⁵ the small quantity of iodine present in the waters of Salzomaggiore is wholly present in combination with organic matter.³⁶

Gautier has also examined the iodine content of air,³⁷ and has shown that neither iodides nor volatile iodine (element or compounds) is present, but that when air is collected over the surface of the sea, and filtered, the residue, consisting of various organisms, shows distinct traces of iodine, while less, but still measurable traces are found in such residues collected by filtering large amounts (3000 liters) of the air over Paris.

Iodine content of plants.

The following analyses are of Green, Brown, and Red Algae, and of the flowering plant, *Zostera marina*, collected near Nanaimo, B. C., during the month of August, 1913. The plants (and the animal material enumerated later) were obtained at the following points:

(a) At the Biological Station, Departure Bay, or at points within half a mile of it.

(b) North-west of the Station, in the neighborhood of Hammond Bay and the "Lagoon."

(c) Near Snake Island, two miles east of the Station.

(d) Near Protection Island, two miles southeast of the Station.

(e) In False Narrows, about eight miles southeast of the Station.

³⁵ Montanari: *L'Orosi*, xxiv, p. 223, 1901.

³⁶ Sonstadt: *Chem. News*, xxv, p. 196, 1892, considered that any inorganic iodine present at the surface of sea-water must be present as iodate, and claimed to have found iodate present.

³⁷ Gautier: *Compt. rend. de l'Acad. des Sci.*, cxxviii, p. 643, 1899.

(f) North of Breakwater Island, two miles east of False Narrows.

(g) On Mudge Island, two miles south of False Narrows.

Material dredged was obtained as a rule between 5 and 15 fathoms.

The sea-water in this district has an extremely low specific gravity, due chiefly to the influx of large bodies of fresh water, such as the Fraser River, into the Straits of Georgia. Daily readings of the specific gravity of the water at the Station, for a period of more than a month, gave an average of 1.015, varying between the limits 1.008 and 1.019. A few readings were made at the other points indicated and were usually nearer the higher limit, varying between 1.015 and 1.017. It is probable that the salinity and iodine content of the sea-water are correspondingly low in these localities.

The Algae were air-dried, further dried over sulphuric acid, and finally heated in a water-oven at 100°C. to constant weight. The results of the analyses follow:

I. ALGAE.

(1) Sub-class *Chlorophyceae*, family *Ulvaceae*. A large number of complete plants were taken in each case, so that the results can be regarded as a fair average.

SPECIES	WHERE OBTAINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
		gram	gram	
<i>Ulva lactuca</i> (? var. <i>latis-</i> <i>sima</i>)	(a); dredged	0.500	0.000103	0.021
<i>Enteromorpha compressa</i>	(a); at low tide	0.500	0.000043	0.009
		0.500	0.000045	0.009
				Mean 0.009
	(f); at low tide	0.200	0.000006	0.003
		0.197	0.000006	0.003
				Mean 0.003
<i>Monostroma fuscum</i>	(a); at low tide	0.500	0.000024	0.005
	(f); at low tide	0.500	0.000021	0.004

(2) Sub-class *Phaeophyceae*. i. Family *Ectocarpaceae*, sp. *Desmarestia ligulata*. A single specimen.

<i>Desmarestia ligulata</i>	(f); dredged	0.500	0.000171	0.034
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ii. Family *Laminariaceae*. The analyses were carried out on single plants and parts of the same plant.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
			gram	gram	
Agarum fimbriatum....	(f); dredged	Frond	0.500	0.000112	0.022
Laminaria saccharina..	(f); dredged	Frond	0.500	0.000770	0.154
			0.500	0.000790	0.158
					Mean 0.156
		Stipe and holdfast	0.500	0.001045	0.209
	(a); just below low water	Frond (small)	0.250	0.000370	0.148
			0.250	0.000411	0.164
					Mean 0.156
	(a); just below low water	Frond (average)	0.2002	0.000354	0.177
	(a); just below low water	Frond (sample of three)	0.500	0.000895	0.179
Laminaria bullata.....	(f); dredged	Frond	0.500	0.000300	0.060
Nereocystis lütkeana...	(a); small	Frond	0.500	0.000920	0.184
		Float	0.500	0.000602	0.120
		Stipe	0.0825	0.000121	0.147
	(a); average size	Frond	0.500	0.000855	0.171
		Float	0.500	0.000449	0.090
		Stipe	0.500	0.000804	0.161
		Holdfast	0.500	0.000419	0.084
	(d); small	Frond	0.500	0.000321	0.064
			0.500	0.000318	0.064
					Mean 0.064
		Float	0.250	0.000543	0.217
		Stipe	0.498	0.000427	0.085
		Holdfast	0.500	0.000528	0.105
			0.399	0.000413	0.103
					Mean 0.104
	(d); average	Frond	0.500	0.000649	0.130
		Float	0.200	0.000216	0.108
		Stipe	0.500	0.000229	0.046
		Holdfast	0.500	0.000855	0.171
	(f); small	Frond	0.500	0.000801	0.160
		Float	0.500	0.000058	0.011

Distribution of Iodine

iii. Family *Fucaceae*. The whole plant was taken, and each sample analyzed was taken from a number of plants.

SPECIES	WHERE OBTAINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE	AVER- AGE
		gram	gram		percent
<i>Fucus evanescens</i>	(a); between tides	0.500	0.000093	0.019	
		0.500	0.000094	0.019	0.019
	(a); between tides	0.500	0.000063	0.013	0.013
	(f); between tides	0.500	0.000040	0.008	
		0.500	0.000042	0.008	0.008
<i>Fucus furcatus</i>	(a); between tides	0.500	0.000087	0.017	0.017
	(a); between tides	0.500	0.000071	0.014	
		0.500	0.000063	0.013	0.013
	(d); between tides	0.500	0.000129	0.026	
		0.500	0.000130	0.026	0.026

(3) Sub-class *Rhodophyceae*. i. Family *Nemalionaceae*. A number of specimens of *Gelidium amansii* were sampled, the whole plant being taken.

SPECIES	WHERE OBTAINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
		gram	gram	
<i>Gelidium amansii</i>	(a); dredged	0.400	0.000369	0.092

ii. Family *Gigartinaceae*. A single plant of *Gigartina radula* (*Gigartina exasperata*) was examined; a number of specimens supposed to be *Gigartina mamillosa* were sampled.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
			gram	gram	
<i>Gigartina radula</i>	(f); low tide	Frond	0.500	0.000037	0.007
		Frond with- out papil- lae	0.500	0.000032	0.006
		Papillae	0.250	0.000015	0.006
<i>Gigartina mamillosa</i> (?)	(f); low tide	Whole plant	0.499	0.000082	0.016
			0.250	0.000038	0.015
					Mean 0.016

iii. Family *Rhodymeniaceae*. A number of specimens of the species examined were sampled.

SPECIES	WHERE OBTAINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
		gram	gram	
<i>Rhodymenia</i> (? <i>corallina</i>)..	(a); dredged	0.500	0.000612	0.122

iv. Family *Rhodomeliaceae*. Samples of a number of specimens of one species were examined, the whole plant being taken.

<i>Rhodomela larix</i>	(f)	0.500	0.000073	0.014
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v. Family *Delesseriaceae*. Samples from several plants in each case.

<i>Nitophyllum</i>	(e); dredged	0.1000	0.000155	0.155
<i>ruprechtianum</i>		0.1500	0.000241	0.161
				Mean 0.158
<i>Nitophyllum violaceum</i> ...	(f); dredged	0.500	0.000636	0.127

vi. Family *Cryptonemiaceae*. Samples from a number of plants in each case.

<i>Prionitis lyallii</i>	(a); dredged	0.500	0.000216	0.043
<i>Halosaccion glandiforme</i> ..	(f); between tides	0.500	0.000029	0.006
<i>Corallina officinalis</i>	(f); between tides	0.500	0.000028	0.006
		0.500	0.000024	0.005
				Mean 0.005

vii. Family *Bangiaceae*. The fronds of single plants of *Porphyra vulgaris* (*Porphyra laciniata*) were examined.

<i>Porphyra vulgaris</i>	(a); below low water	0.500	0.000057	0.011
	(a); below low water	0.500	0.000026	0.005
		0.500	0.000030	0.006
				Mean 0.005
	(d)	0.500	0.000047	0.009
	(f); dredged	0.500	0.000056	0.011

II. FLOWERING PLANT.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PER CENT. IODINE
			<i>gram</i>	<i>gram</i>	
Zostera marina.....	(a); below low water mark	Blades	0.500	0.000015	0.003
			0.500	0.000007	0.001
		Stalk	0.300	0.000010	Mean 0.002
			0.300	0.000005	0.003
		Roots			0.002
			0.1500	0.000019	Mean 0.002
			0.1000	0.000014	0.013
					0.014
					Mean(0.013)

These results are compared with previously published figures in the following table. Much of the data which have been published for Algae cannot be included in this, since they either do not refer to single species, or are expressed for weight of fresh material or for ash. Reference to some of these is made later. In the table the iodine content refers to dry material. Under "Source," "F. W." indicates a fresh water medium, "S. W." a sea-water medium. The method employed by the different observers is indicated after their names, in brackets. Under remarks "Av." indicates average of more than one sample.

SPECIES	SOURCE	PERCENT IODINE	OBSERVER AND REMARKS
<i>Thallophyta</i>			
<i>Bacteria</i>			
Beggiatoa.....	Luchon; sulphur springs	0.036	Gautier ²³ (Bt)
B. diptheriae.....		0	Gautier (Bt)
(Tetanus bacteria).....		0.0002(?)	Gautier (Bt)
<i>Diatomaceae</i> (Diatoms).....		?	Gautier ²³ (Bt)
<i>Cyanophyceae</i> (Blue Algae)			
Nostocaceae			
Nostoc fragilis.....	France; F. W.	0.000423	Gautier ²³ (Bt)
Rivulariaceae.....	Nantes, F. W.	0.000252	Gautier (Bt)
<i>Chlorophyceae</i> (Green Algae)			
Confervaceae			
Ulothrix dissecta.....	France; F. W.	0.00240	Gautier ²³ (Bt)
Cladophora fracta.....	Paris, F. W.	0.000984	Gautier (Bt)
Protococcaceae			
Protococcus pluvialis.....	France; F. W.	0.00206	Gautier (Bt)
Cladophoraceae			
Cladophora glomerata.....	Europe; F. W.	0.0227	Zenger ⁴² (?)
Ulvaceae			
Ulva lactuca (var. latissima ?).....	Nanaimo, B. C., S. W.	0.021	Cameron (H)
Enteromorpha compressa.....	Nanaimo, B. C., S. W.	0.006	Cameron (H)
Monostroma fuscum.....	Nanaimo, B. C., S. W.	0.004	Cameron (H) Av.
<i>Phaeophyceae</i> (Brown Algae)			
Ectocarpaceae			
Desmarestia ligulata.....	Washington, U. S. A., S. W.	0.09	Turrentine ⁴⁴ (Bn) Av.
Desmarestia ligulata.....	Nanaimo, B. C., S. W.	0.034	Cameron (H)
Chordaria flagelliformis.....	Great Britain, S. W.	0.2310	Stanford ⁴⁴ (?)
Laminariaceae			
Pleurophycus gardneri.....	Washington, U. S. A., S. W.	0.12	Turrentine ⁴⁴ (Bn) Av.
Cymathære triplicata.....	Washington, U. S. A., S. W.	0.03	Turrentine ⁴⁴ (Bn) Av.
Costaria turneri.....	U. S. A., S. W.	trace	Turrentine ⁴⁴ (Bn) Av.
Dictyneuron californium.....	California, U. S. A., S. W.	0.09	Turrentine (Bn)
Postelsia palmaeformis.....	U. S. A., S. W.	0.14	Turrentine (Bn) Av.
Agarum fimbriatum.....	Washington, U. S. A., S. W.	0.09	Turrentine (Bn) Av.
Agarum fimbriatum.....	Nanaimo, B. C., S. W.	0.022	Cameron (H)
Laminaria digitata.....	Europe, S. W.	0.135	Sarphat ⁴⁰ (?)
Laminaria digitata.....	Europe, S. W.	0.625	Godechens ⁴⁰ (?)
Laminaria digitata.....	Europe, S. W.	0.444	Wallace ⁴⁰ (?)
Laminaria digitata.....	Great Britain, etc., S. W.	0.2946	(fronds) Stanford ⁴⁴ (?) Av.
Laminaria digitata.....	Great Britain, etc., S. W.	0.4535	(stipes) Stanford (?) Av.
Laminaria digitata.....	W. Schleswig-Holstein, S. W.	0.59	(fronds) Eschle ⁴³ (?)
Laminaria digitata.....	W. Schleswig-Holstein, S. W.	0.19	(stipes) Eschle ⁴³ (?)
Laminaria saccharina.....	Europe, S. W.	0.230	Sarphat ⁴⁰ (?)
Laminaria saccharina.....	Europe, S. W.	3.850	Schweizer ⁴⁰ (?)
Laminaria saccharina.....	Europe, S. W.	0.288	Wallace ⁴⁰ (?)
Laminaria saccharina.....	Europe, S. W.	0.2974	Stanford ⁴⁰ (?)
Laminaria saccharina.....	Washington, U. S. A., S. W.	0.31	Turrentine ⁴⁴ (Bn) Av.
Laminaria saccharina.....	Nanaimo, B. C., S. W.	0.167	(fronds) Cameron (H) Av.
Laminaria saccharina.....	Nanaimo, B. C., S. W.	0.209	(stipes) Cameron (H)
Laminaria bullata.....	Washington, U. S. A., S. W.	0.36	Turrentine ⁴⁴ (Bn) Av.
Laminaria bullata.....	Nanaimo, B. C., S. W.	0.060	Cameron (H)
Laminaria andersonii.....	California, U. S. A., S. W.	0.6	Turrentine ⁴⁴ (Bn)
Nereocystis lütkeana.....	Pacific Coast, U. S. A., S. W.	0.117	Balch ⁴⁴ (?)

SPECIES	SOURCE	PERCENT IODINE	OBSERVER AND REMARKS
<i>Thallophyta</i> (Continued)			
<i>Phaeophyceae</i> (Continued)			
<i>Nereocystis lütkeana</i>	Pacific Coast, U. S. A., S. W.	0.21	(fronds) Parker and Lindemuth ⁴⁵ (Bn) Av.
<i>Nereocystis lütkeana</i>	Pacific Coast, U. S. A., S. W.	0.25	(stipes and holdfast) Parker and Lindemuth ⁴⁵ (Bn) Av.
<i>Nereocystis lütkeana</i>	California, U. S. A., S. W.	0.12	(fronds) Turrentine ⁴⁴ (Bn)
<i>Nereocystis lütkeana</i>	California, U. S. A., S. W.	0.15	(stipes) Turrentine ⁴⁴ (Bn)
<i>Nereocystis lütkeana</i>	Washington, U. S. A., S. W.	0.16	(fronds) Turrentine ⁴⁴ (Bn) Av.
<i>Nereocystis lütkeana</i>	Washington, U. S. A., S. W.	0.08	(stipes) Turrentine (Bn)
<i>Nereocystis lütkeana</i>	Nanaimo, B. C., S. W.	0.142	(fronds) Cameron (H) Av.
<i>Nereocystis lütkeana</i>	Nanaimo, B. C., S. W.	0.109	(float) Cameron (H) Av.
<i>Nereocystis lütkeana</i>	Nanaimo, B. C., S. W.	0.110	(stipe) Cameron (H) Av.
<i>Nereocystis lütkeana</i>	Nanaimo, B. C., S. W.	0.120	(holdfast) Cameron (H) Av.
<i>Macrocystis pyrifera</i>	Pacific Coast, U. S. A., S. W.	0.020	Parker and Lindemuth ⁴⁵ (Bn) Av.
<i>Macrocystis pyrifera</i>	California, U. S. A., S. W.	0.70	Balch ⁴⁴ (?)
<i>Macrocystis pyrifera</i>	California, U. S. A., S. W.	0.28	Turrentine ⁴⁴ (Bn) Av.
<i>Macrocystis pyrifera</i>	Washington, U. S. A., S. W.	0.21	Turrentine ⁴⁴ (Bn) Av.
<i>Macrocystis pyrifera</i>	Alaska, S. W.	0.23	Turrentine (Bn) Av.
<i>Pelagophycus porra</i>	California, U. S. A., S. W.	0.36	Turrentine (Bn) Av.
<i>Alaria valida</i>	Washington, U. S. A., S. W.	0.08	Turrentine (Bn)
<i>Alaria lanceolata</i>	Alaska, S. W.	0.06	Turrentine (Bn) Av.
<i>Egregia menziesii</i>	California, U. S. A., S. W.	0.12	Turrentine (Bn)
<i>Egregia menziesii</i>	Washington, U. S. A., S. W.	0.07	Turrentine (Bn) Av.
<i>Fuaceae</i>			
<i>Fucus serratus</i>	Europe, S. W.	0.121	Sarphat ⁴⁶ (?)
<i>Fucus serratus</i>	Europe, S. W.	0.058	Schweizer ⁴⁷ (?)
<i>Fucus serratus</i>	Europe, S. W.	0.177	Godechens ⁴⁸ (?)
<i>Fucus serratus</i>	Europe, S. W.	0.0565	Wallace ⁴⁹ (?)
<i>Fucus serratus</i>	Europe, S. W.	0.0856	Stanford ⁴⁹ (?)
<i>Fucus nodosus</i>	Europe, S. W.	0.074	Godechens ⁴⁸ (?)
<i>Fucus nodosus</i>	Europe, S. W.	0.0396	Wallace ⁴⁹ (?)
<i>Fucus nodosus</i>	Europe, S. W.	0.0572	Stanford ⁴⁹ (?) Av
<i>Fucus vesiculosus</i>	Europe, S. W.	0.001	Sarphat ⁴⁶ (?)
<i>Fucus vesiculosus</i>	Europe, S. W.	0.0297	Stanford ⁴⁹ (?) Av
<i>Fucus vesiculosus</i>	W. Schleswig-Holstein, S. W.	0.02	Eschle ⁴⁸ (?)
<i>Fucus vesiculosus</i>	Roscoff, S. W.	0.001	Oswald ⁴⁸ (Bn)
<i>Fucus evanescens</i>	California, U. S. A., S. W.	0.13	Turrentine ⁴⁴ (Bn)
<i>Fucus evanescens</i>	Washington, U. S. A., S. W.	0.03	Turrentine (Bn)
<i>Fucus evanescens</i>	Nanaimo, B. C., S. W.	0.13	Cameron (H) Av
<i>Fucus evanescens</i> (var. macrocephala).....	Alaska, S. W.	0.00	Turrentine ⁴⁴ (Bn)
<i>Fucus evanescens</i> (ag. forma).....	Alaska, S. W.	trace	Turrentine (Bn)
<i>Fucus furcatus</i>	California, U. S. A., S. W.	trace	Turrentine (Bn)
<i>Fucus furcatus</i>	Nanaimo, B. C., S. W.	0.019	Cameron (H) Av.
<i>Cystoseira discors</i>	Gulf of Naples, S. W.	0.0085	(Aug.) Scurti ⁴⁷ (Bt)
<i>Cystoseira discors</i>	Gulf of Naples, S. W.	0.045	(April) Scurti (Bt)
<i>Cystophyllum geminatum</i>	Alaska, S. W.	0.28	Turrentine ⁴⁴ (Bn)
<i>Sargassum linifolium</i>	Gulf of Naples, S. W.	0.017	(Oct.) Scurti ⁴⁷ (Bt)
<i>Sargassum linifolium</i>	Gulf of Naples, S. W.	0.127	(April) Scurti ⁴⁷ (Bt)

SPECIES	SOURCE	PERCENT IODINE	OBSERVER AND REMARKS
<i>Thallophyta</i> (Continued)			
<i>Rhodophyceae</i> (Red Algae)			
<i>Nemalionaceae</i>			
<i>Gelidium Amausii</i>	Nanaimo, B. C., S. W.	0.092	Cameron (H)
<i>Gigartinaceae</i>			
<i>Gigartina spinosa</i>	California, U. S. A., S. W.	trace	Turrentine ^a (Bn)
<i>Gigartina radula</i>	California, U. S. A., S. W.	trace	Turrentine (Bn)
<i>Gigartina radula</i>	Nanaimo, B. C., S. W.	0.007	Cameron (H)
<i>Gigartina (fmamillosa)</i>	Nanaimo, B. C., S. W.	0.016	Cameron (H)
<i>Rhodymeniaceae</i>			
<i>Rhodymenia palmata</i>			
<i>linearis</i>	Alaska, S. W.	0.00	Turrentine ^a (Bn)
<i>Rhodymenia (?corallina)</i>	Nanaimo, B. C., S. W.	0.122	Cameron (H)
<i>Rhodomeliaceae</i>			
<i>Rhodomela larix</i>	Nanaimo, B. C., S. W.	0.014	Cameron (H)
<i>Rhodomela pinnastroides</i> ...	Great Britain, S. W.	0.0378	Stanford ^a (?)
<i>Delesseriaceae</i>			
<i>Nitophyllum ruprecht-eaunum</i>	Nanaimo, B. C., S. W.	0.158	Cameron (H)
<i>Nitophyllum violaceum</i>	Nanaimo, B. C., S. W.	0.127	Cameron (H)
<i>Cryptonemiaceae</i>			
<i>Prionitis lyallii</i>	Nanaimo, B. C., S. W.	0.043	Cameron (H)
<i>Halosaccion glandiforme</i>	Nanaimo, B. C., S. W.	0.006	Cameron (H)
<i>Halosaccion glandiforme</i> ...	Alaska, S. W.	trace	Turrentine ^a (Bn)
<i>Corallina officinalis</i>	Nanaimo, B. C., S. W.	0.006	Cameron (H)
<i>Bangiaceae</i>			
<i>Porphyra vulgaris</i>	Nanaimo, B. C., S. W.	0.009	Cameron (H) Av.
<i>Basidiomycetes</i> (Mushrooms)			
<i>Agaricus campestris</i>		0.00027	Bourcet ¹¹ (Bt)
<i>Boletus edulis</i>		Present	Bourcet (Bt)
<i>Cantharellus cibarius</i>		Present	Bourcet (Bt)
<i>Ascomycetes</i>			
<i>Ascolichenes</i> (Lichens)			
<i>Parmelia</i>	Tyrol, 800 metres	trace	Gautier ¹¹ (Bt)
<i>Peltigera</i>	Tyrol 1500 metres	0.000298	Gautier (Bt)
<i>Angiospermae</i>			
<i>Monocotylae</i>			
<i>Naiadaceae</i>			
<i>Zostera marina</i>	Europe, S. W.	0.0005	Sarphat ¹⁰ (?)
<i>Zostera marina</i>	Great Britain, S. W.	0.0378	Sanford ^a (?)
<i>Zostera marina</i>	Nanaimo, B. C., S. W.	0.002	Cameron (H)
<i>Liliaceae</i>			
<i>Allium sativum</i> (garlic).....	France	0.000094	Bourcet ¹¹ (Bt)
<i>Allium cepa</i> (onion).....	France	0.000023	Bourcet (Bt)
<i>Allium porrum</i> (leek).....	France	0.000012	Bourcet (Bt)
<i>Dicotylae</i>			
<i>Choripetalae</i>			
<i>Polygonaceae</i>			
<i>Rumex acetosa</i> (sorrel)....	France	0.0000047	Bourcet (Bt)
<i>Chenopodiaceae</i>			
<i>Beta rapa</i> (red beet).....	France	0.000014	Bourcet (Bt)
<i>Beta cycla</i> (beet).....	France	0.000038	Bourcet (Bt)
<i>Spinacea oleracea</i>			
(spinach).....	France	0.0000021	Bourcet (Bt)

SPECIES	SOURCE	PERCENT IODINE	OBSERVER AND REMARKS
<i>Angiospermae</i> (Continued)			
<i>Dicotylae</i> (Continued)			
Cruciferae			
Raphanus sativus (radish)	France	0.000018	Bourcet (Bt)
Raphanus (Sp.?) (black radish).....	France	0.000000	Bourcet (Bt)
Brassica napus (turnip)...	France	0.000024	Bourcet (Bt)
Brassica (Sp. ?) (long radish).....	France	0.000016	Bourcet (Bt)
Umbelliferae			
Scandix cerforium.....	France	0.000014	Bourcet (Bt)
Petroselinum sativum (parsley).....	France	0.000000	Bourcet (Bt)
Daucus carotta (carrot)...	France	0.000000	Bourcet (Bt)
Leguminosae			
Pisum sativum (green pea)	France	0.0000084	Bourcet (Bt)
Phaseolus (green haricot).	France	0.000032	Bourcet (Bt)
Phaseolus (Sp. ?, of Poisons).....	France	0.0000013	Bourcet (Bt)
Faba vulgaris (bean).....	France	0.000014	Bourcet (Bt)
Symptetales			
Solanaceae			
Solanum tuberosum (potato).....	France	0.0000000	Bourcet (Bt)
Solanum melongena (egg plant).....	France	0.000001	Bourcet (Bt)
Lycopersicum esculentum (tomato).....	France	0.000007	Bourcet (Bt)
Cucurbitaceae			
Cucumis sativus (cucumber).....	France	0.0000012	Bourcet (Bt)
Cucumis sativus (gherkin)	France	0.000000	Bourcet (Bt)
Cucumis melo (melon).....	France	0.000006	Bourcet (Bt)
Cucurbita maximus (pumpkin).....	France	0.0000017	Bourcet (Bt)
Compositae			
Lactuca sativa (lettuce)...	France	0.0000096	Bourcet (Bt)
Cichorium intybus (chicory).....	France	0.0000000	Bourcet (Bt)
Cichorium augustifolium (endive).....	France	0.0000000	Bourcet (Bt)

³⁸ Gautier: *Compt. rend. de l'Acad. des Sci.*, cxxix, p. 189, 1899.

³⁹ Gautier: *ibid.*, cxxviii, p. 1069, 1899.

⁴⁰ Quoted by Stanford: *Chem. News*, xxxv, p. 172, 1877.

⁴¹ Turrentine: Document 190, 62d Congress, 2d Session, 1912, p. 220.

⁴² Stanford: *Chem. News*, xxxv, p. 172, 1877.

⁴³ Eschle: *Zeitschr. f. physiol. Chem.*, xxiii, p. 30, 1897.

⁴⁴ Balch: *Journ. of Ind. and Eng. Chem.*, i, p. 777, 1909.

⁴⁵ Parker and Lindemuth: *ibid.*, v, p. 287, 1913.

⁴⁶ Oswald: *Zeitschr. f. physiol. Chem.*, lxxv, p. 353, 1911.

⁴⁷ Scurti: *Gazz. chim. ital.*, xxxvi, (ii) p. 619, 1906.

⁴⁸ Bourcet: *Comp. rend. de l'Acad. des Sci.*, cxxix, p. 768, 1899. (Bourcet's figures presumably refer to dried material.)

The following data could not be included in the above table, since they do not refer to dry tissue. Yniestra⁴⁹ found that iodine is present in the Mexican "sabilla," a kind of aloe, growing on plains and the slopes of mountains, and in "los romeritos," a kind of barilla, growing on the floating gardens of fresh-water lakes near Mexico City, and much eaten during "la carême." Allary,⁵⁰ working with fresh material, found that iodine varied in content between the limits 0.01 and 0.1 per cent in different species of Laminaria. He concluded that there are variations in different species, in the same species, and with latitude. From experiments with *Digitatus stenolobus* he concluded that when a leaf ceases to grow, and commences to lose its vitality, it gives up iodine to the surrounding liquid. Tunmann⁵¹ reported that the leaf of Laminaria contains more iodine than the stipe. C. J. White⁵² found 0.89 per cent iodine in the ash of *Ecklonia exasperata* (Laminariaceae), the common brown sea-weed found below low tide mark on the New South Wales coast. Harnack states⁵³ that it is commonly considered that the tobacco leaf contains iodine.

In connection with the results of Bourcet quoted in the above table, it should be stated that they were obtained for vegetables growing under precisely similar conditions, in earth containing 0.83 mgm. iodine per 100 kilograms (0.00000083 per cent). Bourcet concluded from these results that under identical conditions of soil, humidity, and exposure, certain plants absorb more iodine than others, others absorb none.

Scurti's results on *Sargassum linifolium* and *Cystoseira discors* were carried out over the whole year, and indicated a gradual continuous change, the extremes being obtained in April, and in the autumn, as shown in the table. Scurti concluded that iodine holds in Algae the place held by chlorine in the Phanerogams, and that it acts therefore as an excitant of the reproductive phase.

⁴⁹ Yniestra: *Ann. chim. phys.*, (ii) lxii, p. 110, 1836.

⁵⁰ Allary: *Bull. soc. chim. Paris*, xxxv, p. 11, 1881.

⁵¹ Tunmann: *Pharmaceut. Centralh.*, xlviii, p. 505, 1907.

⁵² C. J. White: *Journ. Roy. Soc. N.S.W.*, xli, p. 95, 1907.

⁵³ Harnack: *Munch. med. Wochenschr.*, xliii, p. 196, 1896.

The data for plants given in the above table are sufficiently complete to show that iodine is present in measurable amount in the great majority of plants. The amount present in the environment is undoubtedly the chief determining factor of the amount present in the plant, as indicated by the fact that for fresh-water Algae, and for vegetables, the figures are of a second order lower than for marine Algae. The table shows conclusively that closely related species have, however, markedly different selective affinities for the element. This is especially illustrated by Bourcet's figures for vegetables grown under the same conditions; my own figures for Algae from the same locality also show this difference. The variation in the same species under closely approximating conditions is well shown by my results for plants from different localities within a small area. These variations are indicated to a more marked extent by the summarized results in the tables, and are extremely well shown in the original tables for *Macrocystis* and for *Nereocystis* of Turrentine, and of Parker and Lindemuth. How far these variations are due to slight differences of iodine content in the medium is not at present clear, and more observations are required to throw light upon this question. My results seem to show that generally speaking, plants growing above low water mark contain percentages of iodine of a lower order than those growing below this limit.

Allary suggests that latitude is a factor. Parker and Lindemuth consider that there is no difference in iodine content in the giant kelps growing off the north and south Pacific coasts. For one or two of the Brown Algae data are available for California, Washington, Nanaimo, B. C., and Alaska, and these do seem to indicate that the iodine content gets less at higher latitudes. The Nanaimo results, however, which are almost invariably less than those obtained for the same species further south, are, as I have indicated at the beginning of this section, for plants growing under probably abnormal conditions as regards salinity and iodine content of the medium. I am not satisfied as to the extent of possible variations in plants growing under precisely the same conditions, and hope to test this point further. It is clear that different parts of the same plant may contain different amounts of the element. This is well shown by the results for *Nereocystis*.

The results here, as in other species for which observations have been made, are conflicting, and further work is required to determine whether there is any law governing the distribution of iodine within the plant.

Allary, as already mentioned, states that when a leaf of one of the marine Algae ceases to grow, and commences to lose its vitality, it gives up iodine to the surrounding medium. I have made a few observations bearing on this point which perhaps can be best given here.

Two analyses on 0.5 gram of *Corallina officinalis* gave, as shown above (p. 347), the respective figures 0.005 and 0.006 per cent. Two analyses on specimens of the same plant, found in the same locality, but shrunk by exposure, gave also the figures 0.005 and 0.006 per cent, that is, there was no difference within the limit of error of experiment. The amount of iodine, it will be observed, was very small.

Agarum fimbriatum was found to contain 0.022 per cent (p. 345). A bleached sample, found cast up on the beach in the same locality, contained 0.014 per cent.

Three plants of *Laminaria saccharina* were sampled, and the almost complete plants were then placed in distilled water, and left for a week. They were then again sampled, and the samples analyzed. The respective figures were 0.179 and 0.033 per cent.

These results support Allary's conclusion.

(Zenger's figure for *Cladophora*, and Schweizer's, for *Laminaria saccharina*, are almost certainly incorrect.)

Iodine Content of Animals.

The Nanaimo material, the analyses of which follow, was collected during August, 1913, and (except in the case of a few shells and tests which were air-dried) was preserved either in absolute alcohol, or in a few cases in dilute formaldehyde. In all cases before analysis the alcohol (or formaldehyde) was evaporated, and the material heated to constant weight in a water-oven at 100° C., so that the results are all expressed for dry tissue. The dog and rabbit tissue, and other material obtained in the laboratory, were dried in the water-oven as soon as obtained.

It will be convenient to deal with each phylum separately, giv-

ing first my own results, and then a comparison of these with those previously published.

(A) *Protozoa*. Gautier's results⁵⁴ suggest that flagellates and rotiferas may contain iodine.

(B) *Metazoa*. (1) Phylum *Porifera*. I have examined six species of sponges, one calcareous, *Aprocallistes*, and five non-calcareous. Single specimens were examined in each case.

SPECIES	SOURCE	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
		gram	gram	
Hexactinellida				
Aprocallistes whiteavesianus.....	(c); dredged	0.500	0.000097	0.019
Rhabdocalyptus dowingii.....	(c); dredged	0.548	0.000075	0.014
Bathydorus dawsonii....	(c); dredged	0.499	0.000045	0.009
Monaxonida				
Myxilla parasitica.....	(c); dredged	0.500	0.000049	0.010
Esperella adhaerens.....	(c); dredged	0.501	0.000073	0.015
		0.501	0.000074	0.015
				Mean 0.015
Reniera rufescens.....	(a); at very low tide	0.500	0.000058	0.012

The number of exact observations on the dried tissue of sponges is not very numerous. Croockewit⁵⁵ found ordinary bath sponge to contain after extraction with ether, alcohol, and water, 1.1 per cent. Harnack⁵⁶ found in the same substance 1.1 to 1.2 per cent (1.5 to 1.6 per cent after removing inorganic material). Proust⁵⁷ found 1.8 per cent for the organic raw substance. Hundeshagen⁵⁸ found that tropical and subtropical sponges contain higher percentages. Thus many species of *Luffaria*, *Euchalina*, and *Verongia*,

⁵⁴ Gautier: *Compt. rend. de l'Acad. des Sci.*, cxxviii, p. 1069, 1899.

⁵⁵ Croockewit: *Ann. der Chem. u. Pharm.*, xlviii, p. 43, 1843.

⁵⁶ Harnack: *Zeitschr. f. physiol. Chem.*, xxiv, p. 412, 1898.

⁵⁷ Proust, cited by Pereira: *Buchheim's Handbuch der Heilmittellehre*, Leipzig, 1848, i, p. 824.

⁵⁸ Hundeshagen: *Zeitschr. f. angew. chem.*, 1895, p. 473; quoted through Abderhalden's *Handbuch der Biochemie*, iv, p. 169.

contained from 8 to 14 per cent. The iodine content of *Euspongia*, *Cholinopsis*, and the *Aplysinidae* of the Mediterranean is smaller.

It is evident that the Nanaimo sponges contain relatively little iodine.

(2) Phylum *Coelenterata*. I have examined several species in this phylum. The specimens of *Obelia* were attached to the wharf at the Biological Station. They were washed free from dirt, and preserved in alcohol. Foreign organisms present (diatoms, ostracods, caprellae) certainly did not amount to one per cent of the total weight. A number of *Aequorea* were obtained in False Narrows, and the *Aurelia* were obtained in the same locality. The sea-anemones were obtained on rocks near the Station. The complete animals could not be removed from the rocks, but the larger part was removed by cutting as the animal hung above low water. The comb-jellies, probably a species of *Pleurobrachia*, were obtained near the Station. These four species were preserved in dilute formaldehyde. Their weights, after hardening by the formaldehyde, were determined, and the whole evaporated to dryness. The "formaldehyde" and dry weights are quoted, although I do not know in how far the original weight was altered by the addition of the formaldehyde. The dried material appeared to consist chiefly of crystalline salts. Some of the iodine, if present, may have been lost by the evaporation of what was initially a slightly acid solution.

There are no corals obtainable in the Nanaimo district.

CLASS	SPECIES	(FRESH) WEIGHT	DRY WEIGHT	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
		grams	grams	gram	gram	
Hydrozoa.....	<i>Obelia longissima</i>			0.500	0.000067	0.013
				0.500	0.000064	0.013
				0.500	0.000066	0.013
					Mean	0.013
	<i>Aequorea forskalia</i>	317	17.20	0.500	0	0.000
Scyphozoa.....	<i>Aurelia flavidula</i>	158	9.96	0.500	0	0.000
Actinozoa.....	<i>Matridium marginatum</i>	83	7.74	0.500	0	0.000
Ctenophora.....	<i>Pleurobrachia</i> (Sp.?)			0.500	0	0.000

A. B. Macallum⁵⁹ examined the fresh tissue of *Aurelia flavidula*, using Bourcet's method of analysis, and found a content of 0.0000005 per cent iodine. The sea-water in which the specimens were obtained contained 0.000001 per cent of the element.

Numerous observations have been made on corals, some species of which contain relatively large amounts of iodine. The figures published are all given for the organic substance of the skeleton, "gorgonin," freed as far as possible from other tissue and from inorganic material. The figures are therefore not comparable with those given elsewhere in this paper.

Drechsel⁶⁰ was the first to observe the presence of iodine in gorgonin. He obtained 7.789 per cent in the gorgonin from *Gorgonia cavolinii*. L. B. Mendel⁶¹ confirmed the presence of the element. Cook examined a large number of West Indian Corals,⁶² with invariably positive results. Mörner has carried out a very large number of analyses.⁶³ He finds that iodine is an invariable constituent of the organic skeleton-substance, in amounts varying from traces up to seven per cent. For the same species the iodine content is relatively constant, and seems to be independent of climate.

(3) Phylum *Vermes*, sub-phylum *Annulata*, class *Chaetopoda*, order *Polychaeta*. I have examined several annelids. It will be observed that the results for the tubes of some species are distinctly high. I have been unable, so far, to obtain definite identification of all the species examined. The worms were preserved in alcohol, the tubes air-dried.

⁵⁹ Macallum: *Journ. of Physiol.*, xxix. p. 213, 1903.

⁶⁰ Drechsel: *Zeitschr. f. Biol.*, xxxiii, p. 90, 1896.

⁶¹ Mendel: *Amer. Journ. of Physiol.*, iv, p. 243, 1901.

⁶² Cook: *ibid.*, xii, p. 95, 1905.

⁶³ Mörner: *Zeitschr. f. physiol. Chem.*, li, p. 33, 1907; lv, pp. 77, 223, 1908.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE	AVER- AGE
			gram	gram		
A <i>Nereis</i> worm.....	(g); at low tide	Worm	0.500	0.000043	0.009	0.008
			0.500	0.000035	0.007	
	(b); at low tide	Worm	0.500	0.000094	0.019	0.017
			0.500	0.000082	0.016	
A <i>Nephtys</i> worm.....	(g); at low tide	Worm	0.400	0.000035	0.009	0.009
Diopatra (Sp. ?).....	(g); at low tide	Worm	0.500	0.000124	0.025	0.023
			0.500	0.000109	0.022	
			0.500	0.000115	0.023	
		Inner tube	0.300	0.001247	0.416	0.414
			0.1000	0.000411	0.411	
		Outer tube	0.500	0.001358	0.272	
			0.300	0.000741	0.247	
	(a); at low tide	Worm	0.500	0.000192	0.038	0.039
			0.500	0.000198	0.040	
			0.500	0.000189	0.038	
		Tube	0.500	0.000159	0.032	0.030
			0.500	0.000156	0.031	
			0.500	0.000137	0.027	

The calcareous tube of the Serpulid worm was submitted to a further rough examination.

10.00 grams of the powdered dry substance were treated with cold dilute hydrochloric acid for 24 hours, and the residue filtered, washed, and dried at 100°. It weighed 0.512 gram. Of this 0.1015 gram after ignition left a residue of 0.0535 gram, while 0.1000 gram contained 0.000351 gram iodine (0.351 per cent). Hence, assuming that the whole of the iodine of the residue was in organic combination, and not in the ash, since the original material contained 0.003 gram, and the residue 0.0018 gram, it follows that the larger part, if not all of the iodine was in organic combination, and to the extent of at least 0.74 per cent, an amount of the same order of magnitude as that found for the *Diopatra* tube.

The outer tube of *Diopatra* included layers of both secretions (lip and tori). The inner tube was the parchment tube secreted by the tori.

Sarphati⁶⁴ reported iodine present in turbellarian worms. The only other observation on Annelids that I have found is that of Mörner,⁶⁵ who found that the tubes of *Chaetopterus norvegicus* and *Hyalinaecia tubicola* contained, after careful removal of calcium carbonate, 0.22 and 0.09 per cent of iodine, respectively. Since these tubes should not contain much calcium carbonate, these numbers are probably directly comparable with those obtained for *Diopatra*.

I have found no exact data for the phyla Molluscoida, Echinodermata, Arthropoda, and Mollusca, and none for the sub-phylum Tunicata. According to Balard,⁶⁶ iodine is present in oysters. Sarphati⁶⁷ found it in *Asterias rubens*, *Crognon vulgare*, and *Mytilus edulis*. Rieger⁶⁸ found it in crabs.

I have examined representatives of each of the phyla just mentioned, with the following results:—

(4) Phylum Molluscoida, class Polyzoa, family Cellularina. The specimens of the species examined were obtained on a plant of *Laminaria bullata* dredged in Departure Bay, Nanaimo. They were washed free from adhesive material (examination under the microscope revealing the presence of only a few foreign forms) and were preserved in absolute alcohol.

SPECIES	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
	gram	gram	
Bugula flabellata.....	0.2500	0.000039	0.016
	0.1000	0.000017	0.017
			Mean 0.016

⁶⁴ Sarphati: *Repert. f. die Pharm.*, lix, p. 314, 1850 (?), quoted through Gmelin's *Handbuch der anorgan. Chem.*, i, Abt. 2, p. 287.

⁶⁵ Mörner: *Zeitschr. f. physiol. Chem.*, lv, p. 83, 1908.

⁶⁶ Balard: quoted through Gmelin's *Handbuch*, loc. cit.

⁶⁷ Sarphati: loc. cit.

⁶⁸ Rieger: *Jahresbuch f. Chem.*, 1853, p. 329; quoted through Gmelin's *Handbuch*, loc. cit.

(5) Phylum *Echinodermata*. i. Class *Echinoidea*.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PER CENT IODINE
			gram	gram	
Strongylocentrotus drobachiensis.....	(e); dredged	Aristotle's Lantern	0.500	0	0.000
		Internal organs	0.0697	0.000014	0.02
		Gonads	0.500	0.000018	0.004
			0.500	0.000015	0.003
					Mean 0.003
Strongylocentrotus franciscanus, var. purple.....	(e); dredged	Test	0.500	0	0.000
		Spines	0.500	0	0.000
		Internal organs	0.250	0.000125	0.050
			0.300	0.000139	0.046
			0.1000	0.000058	0.058
					Mean 0.049
Strongylocentrotus franciscanus, var. red.....	(e); dredged	Gonads	0.500	0.000004	(trace)
		Aristotle's Lantern	0.500	0.000010	0.002
			0.500	0.000007	0.001
					Mean 0.001

ii. Class *Holothuroidea*. A specimen of *Stichopus californensis* (dredged; b), was examined. I am not satisfied with the results, but they indicate that if iodine is present, it is present in relatively very small quantity.

SPECIES	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PERCENT IODINE
		gram	gram	
Stichopus califor- nensis.....	Integument (preserved in alcohol)	0.500	0.000003	0.001
		0.503	0.000018	0.004
		0.250	0.000005	0.002
		0.250	0.000005	0.002
	Muscle			Mean 0.002
		0.1000	0	0.00

iii. Class *Asteroidea*. One complete ray of the whole animal was preserved in alcohol, and a sample of the whole ray examined.

SPECIES	WHERE OBTAINED	AMOUNT TAKEN	IODINE FOUND	PERCENT IODINE
		gram	gram	
<i>Pyknapodia heli-anthoides</i>	(a) Between tides	0.500	0	0.000

(6) Phylum *Arthropoda*, class *Crustacea*. The barnacles, *Balanus*, were attached to piles at the Station wharf; the specimen of *Cancer* examined was obtained in shallow water at the same place.

SPECIES	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PERCENT IODINE
		gram	gram	
<i>Balanus balanoides</i> .	Shell	0.500	0	0.000
	Soft part	0.200	0.000010	0.005
<i>Cancer productus</i> ...	Carapace	0.500	0.000016	0.003
		0.500	0.000015	0.003
				Mean 0.003
	Muscle	0.2000	0	0.00

(7) Phylum *Mollusca*, class *Pelecypoda*.

SPECIES	WHERE OBTAINED	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PERCENT IODINE
			gram	gram	
<i>Mya arenaria</i>	(a); low water	Shell	0.500	0	0.000
		Soft part	0.400	0.000035	0.009
			0.400	0.000035	0.009
					Mean
<i>Schizothoerus nuttalli</i>	(g); low water	Shell	0.501	0	0.000
		Outside cuticle of foot	0.300	0.000893	0.298
		Muscle of foot	0.1995	0	0.00
		Heart and Kidney	0.0350	0.000009	(0.02)
		Gonads	0.500	0	0.000
		Gills	0.2000	0	0.00

A second analysis of the outer cuticle of the foot of *Schizothoerus* indicated a result of the same order but was spoilt before completion. The figure given for heart and kidney requires repetition.

(8) Phylum *Chordata*. i. Subphylum *Tunicata*. Only a few specimens of one form were obtained (at Mudge Island, at very low tide), and these did not yield sufficient material for definite results, except in the case of the test.

SPECIES	PART EXAMINED	AMOUNT TAKEN	IODINE FOUND	PERCENT IODINE
		gram	gram	
Pyura haustor.....	Test	0.300	0.000605	0.202
		0.300	0.000595	0.198
				Mean 0.200
	Inner layer of test	0.1500	0.000016	(0.010)
	Mantle	0.1000	0.000012	(0.012)
	Gonads	0.2500	0	0.00

ii. Sub-phylum *Vertebrata*. Since the results show conclusively that no tissue contains iodine in amounts of the same order of magnitude as the thyroid, it will be convenient to consider first the available data for this gland. Numerous observers claim to have shown the presence of iodine in marked amount in various other organs of internal secretion, so that I shall subsequently deal with these in turn, and then consider finally the figures available for the remaining body tissues.

Thyroid. I have carried out a number of new analyses of thyroid tissues of different animals, with the following results. (In this table, and that following, f=female, m=male.)

SPECIES	NUMBER OF ANIMALS	WEIGHT	THYROID WEIGHT			AMOUNT SUBSTANCE TAKEN	IODINE FOUND	PERCENT IODINE
			Fresh	Dry	Per cent dry wt.			
Squalus sucklii (Elasmobranchii).....	32 (f)	kg.	gram	gram		gram	gram	
	34 (f)			0.659		0.2015	0.000394	0.195
				0.800		0.2003	0.000391	0.195
						0.1005	0.000197	0.196
	16 (m)			0.169		0.1000	0.000224	0.224
	133			0.066		0.0604	0	0.00
Brown rat.....	8	1.5	0.247	0.060	24	0.060	0.000014	0.023
Albino rat (adult).....	2	0.651	0.062	0.016	26	0.016	0.000005	0.033
(young).....	6	1.017	0.087	0.032	37	0.032	0.000035	0.109
Guineapig.....	4	1.869	0.260	0.053	20	0.053	0.000049	0.092
	4	1.674	0.238	0.049	21	0.049	0.000039	0.080
	7	3.085	0.355	0.068	19	0.068	0.000105	0.154
Rabbits.....	4	7.50	0.577	0.215	37	0.215	0.000137	0.064
	1			0.088		0.088	0.000108	0.123
	(?)		0.872	0.167	19	0.167	0.000067	0.040
Cat (adult).....	1	2.5	0.202	0.052	26	0.052	0.000028	0.054
	1	2.75	0.343	0.068	20	0.068	0.000007	0.010
(kitten, $\frac{1}{2}$ grown)...	3	2.0	0.434	0.100	23	0.100	0.000019	0.019
(kitten, 2 weeks)...	2	0.375	0.087	0.019	22	0.019	0.000003	0.016
(kitten, $\frac{1}{2}$ grown)...	1	1.409	0.135	0.044	33	0.044	0.000135	0.307
Dog (adult).....	1 (f)	12.5	2.072	0.569	27.5	0.569	0.000352	0.062
(adult, goitrous)...	1 (m)	18	25.8	8.017	31	0.500	0.000195	0.039
(pup).....	1 (f)	5	0.521	0.159	30.5	0.159	0.000114	0.072
(pup).....	1 (f)	4	0.588	0.160	27	0.160	0.000162	0.101
(pup).....	1 (f)	2.7		0.136		0.136	0.000192	0.141
(pup).....	1 (m)	2.8		0.421		0.421	0.000274	0.065
(pup, goitrous)....	1 (f)	2.7	6.00	1.319	22	0.500	0.000180	0.036

I have found no previous data for the rat. The experiments on cats were carried out on account of Roos' negative values (see below).

The available data for thyroid tissue are summarized in the following table. Where more than one set of analyses are available, and different methods have been employed, the more accurate has been quoted. (Large numbers of figures are available for sheep, cattle, swine, and dogs.)

SPECIES	NUMBER OF ANIMALS	LIMITS OBSERVED FOR IODINE CONTENT	AVERAGE IODINE CONTENT	OBSERVER AND REMARKS
		per cent	per cent	
<i>Pisces</i>				
Scyllium canicula (f)...	large		1.160	Cameron ⁶⁹ (H)
Scyllium canicula (m)	large		0.719	Cameron ⁶⁹ (H)
Squalus acanthias....	large		0.133	Cameron ⁷⁰ (H) Minimum
Squalus sucklii (f)....	66		0.195	Cameron ⁷¹ (H)
Squalus sucklii (m)...	16		0.224	Cameron (H)
Raia clavata.....	large	0.233-0.438	0.404	Cameron ⁶⁹ (H)
<i>Amphibia</i>				
Rana pipiens.....	93		0.063	Cameron ⁷⁰ (H) Minimum
<i>Reptilia</i>				
Alligator.....	3		0.059	Cameron ⁷⁰ (H)
Testudo (African tortoise).....	8		Pres-	
<i>Aves</i>			ent	Doyon and Chenu ⁷² (Bn)
Columba (Pigeon)....	large	0.453-0.550	0.485	Cameron ⁷⁰ (H)
Gallus (Domestic fowl).....	2		Pres-	
<i>Mammalia</i>			ent	Chenu and Morel ⁷³ (Bn)
Equus (Horse).....	3	0.06-0.17	0.12	Baumann ⁷⁴ (Bn)
Sus (Swine).....	large	0.133-0.531	0.230	Seidell and Fenger ⁷⁵ (H)
				Fat-free
Cervus (Roe) (f)....	5	0.02-0.24	0.14	Roos ⁷⁶ (Bn)
(m)....	2		0.24	Roos (Bn)
(Stag) (f)....	9	0.1-0.54	0.21	Blum ⁷⁷ (Bn)
(m)...	2		0.28	Blum (Bn)
Ovis (Sheep).....	large	0.048-0.335	0.167	Seidell and Fenger ⁷⁵ (H)
				Fat-free
	6560	0.30-0.40	0.343	Martin ⁷⁸ (?)
Capra (Goat) (f)....	2		0.16	Blum ⁷⁷ (Bn)
(m)....	7	0.04-0.28	0.18	Blum (Bn)
Bos (Cattle).....	large	0.028-0.361	0.188	Seidell and Fenger ⁷⁵ (H)
				Fat-free
Lepus (Hare).....	14	0.02-0.14	0.06	Roos ⁷⁶ (Bn)
(Rabbit).....	several	0.040-0.123	0.067	Cameron ⁷¹ (H)
Cavia (Guineapig)....	15	0.080-0.154	0.114	Cameron (H)
Mus (Brown Rat)...	8		0.023	Cameron (H)
(Albino Rat)...	2		0.033	Cameron (H)
(Albino Rat Young).....	2		0.109	Cameron (H)
Felis (Domestic Cat)	9	0.00-0.05		Roos ⁷⁶ (Bn)
	2	0.010-0.054	0.029	Cameron ⁷¹ (H)
(Young).....	6	0.016-0.307	0.096	Cameron (H)
(Wildcat).....			0.00	Roos ⁷⁶ (Bn)

SPECIES	NUMBER OF ANIMALS	LIMITS OBSERVED FOR IODINE CONTENT	AVERAGE IODINE CONTENT	OBSERVER AND REMARKS
		per cent	per cent	
<i>Mammalia</i> (Continued)				
<i>Canis</i> (Domestic Dog)	large		0.332	Marine and Lenhart (Bn)
	12	0.011-0.265	0.096	Cameron ⁷¹ (H)
(Young).....	4	0.065-0.141	0.085	Cameron (H)
(Fox).....	3		0.00	Roos ⁷⁶ (Bn)
	11	trace-0.277		Carlson and Woelfel ⁸⁰ (Bn)
<i>Mustela</i> (Polecat)....	1		0.00	Roos (Bn)
(Stone-marten).....	3	0.0-0.2		Roos (Bn)
(Pine-marten).....	2	0.0-trace		Roos (Bn)
<i>Homo</i> (Freiburg) (f)..	12	trace-0.06	0.018	Baumann ⁸¹ (Bn)
(m).	14	trace-0.09	0.036	Baumann (Bn)
(Hamburg) (f).	25	trace-0.21	0.088	Baumann (Bn)
(m)	5	0.06-0.10	0.088	Baumann (Bn)
(Berlin) (f)....	1		0.110	Baumann (Bn)
(m)....	10	0.05-0.14	0.086	Baumann ⁸¹ (Bn)
(Geneva) (f)...	7	0.034-0.153	0.057	Oswald ⁸² (Bn)
(m).	15	0.041-0.190	0.118	Oswald (Bn)
(Lausanne) (f).	4	0.034-0.146	0.123	Oswald (Bn)
(m)	7	0.008-0.160	0.050	Oswald (Bn)
(Basel) (f)....	13	0.038-0.146	0.086	Oswald (Bn)
(m)....	9	0.053-0.177	0.105	Oswald (Bn)
(Zurich) (f)....	9	0.046-0.123	0.093	Oswald (Bn)
(m)...	15	0.011-0.254	0.098	Oswald (Bn)
(Bern) (f).....	10	0.015-0.308	0.129	Oswald (Bn)
(m).....	8	0.046-0.207	0.121	Oswald (Bn)
(Schlesien).....	50		0.057	Weiss ⁸³ (Bn)
(Steiermark)...	19	0.008-0.07	0.032	v. Rozitsky ⁸⁴ (?)

⁶⁹ Cameron: *Biochem. Journal*, vii, p. 466, 1913.

⁷⁰ Cameron: *this Journal*, xvi, p. 467, 1914.

⁷¹ Cameron: *this paper*, see above.

⁷² Doyon and Chenu: *Compt. rend. de l'Acad. des Sci.*, cxxxix, p. 157, 1904

⁷³ Chenu and Morel: *ibid.*, cxxxviii, p. 1004, 1904.

⁷⁴ Baumann: *Zeitschr. f. physiol. Chem.*, xxii, p. 17, 1896.

⁷⁵ Seidell and Fenger: *this Journal*, xiii, p. 517, 1912.

⁷⁶ Roos: *Zeitschr. f. physiol. Chem.*, xxviii, p. 55, 1899.

⁷⁷ Blum: *Archiv f. d. ges. Physiol.*, lxxvii, p. 70, 1899.

⁷⁸ Martin: *Pharmaceut. Journ.*, lxxxix, p. 144, 1912.

In the above table the figures for the dog are taken from Marine and Lenhart's figures for "normal" dogs, and from my own in this and a previous paper,⁷⁰ excluding marked goitres only. The figures for man are calculated from the data given in the original papers of Baumann, Oswald, etc. Although the material was obtained from pathological cases, the thyroids were stated to be normal.

The above table does not include all published data, as I have indicated, so that some of the maximum figures are not included. The maximum figures which I have been able to find are: Fish-thyroids, 1.16 per cent (see table); dog-thyroids, 0.692 per cent (Marine and Lenhart⁸⁵); human-thyroids, 0.588 per cent (Seidell⁸⁶); stag-thyroids, 0.54 per cent (see table), pig-thyroids, 0.531 per cent (see table); sheep-thyroids, 0.53 per cent (Baumann and Roos⁸⁷); beef-thyroids, 0.477 per cent (Marine and Lenhart⁸⁵).

In considering the above tables, it will be remarked that a few of the earlier analyses (Roos) show negative results. I have shown above (p. 338) that this is probably to be attributed to the error of analysis of the method employed, and have given reasons previously⁷⁰ for the statement that iodine is to be considered a normal constituent of all normal thyroid tissue. Examination of the above table fully confirms this view. The amount present, for dry tissue, appears to vary between the limits 0.01 and 1.0 per cent. The numbers indicate no regular relationship to species. This is well shown in the following table, in which the data are taken from the analyses published in this, and in a previous paper,⁷⁰ except in the cases indicated.

⁷⁹ Marine and Lenhart: *Arch. of Int. Med.*, iii, p. 66, 1909.

⁸⁰ Carlson and Woelfel: *Amer. Journ. of Physiol.*, xxvi., p. 32.

⁸¹ Baumann: *Zeitschr. f. physiol. Chem.*, xxii, p. 1, 1896.

⁸² Oswald: *ibid.*, xxiii, p. 265, 1897.

⁸³ Weiss: *Munch. med. Wochenschr.*, xlv, p. 6, 1897.

⁸⁴ v. Rositzky: *Wiener klin. Wochenschr.*, x, p. 823, 1897.

⁸⁵ Marine and Lenhart: *Arch. of Int. Med.*, iv, p. 440, 1909.

⁸⁶ Seidell: this *Journal*, x, p. 95, 1911.

⁸⁷ Baumann and Roos: *Zeitschr. f. physiol. Chem.*, xxi, p. 481, 1895.

ANIMAL	PER CENT IODINE IN THYROID	MILLIGRAMS DRY THY- ROID TISSUE PER KILOGRAM ANIMAL	MILLIGRAM IODINE (IN THYROID) PER KILOGRAM ANIMAL
Dog-fish (<i>Squalus sucklii</i>) (f).....	0.195	6	0.001
(m).....	0.224	4	0.009
Pigeons (24, weighing 6.5 kg.).....	0.477	21	0.099
Rabbits (4, weighing 7.5 kg.).....	0.064	29	0.086
Guineapigs (15, weighing 6.62 kg.).....	0.113	26	0.028
Brown rats (8, weighing 1.5 kg.).....	0.023	40	0.009
Albino rats (2, weighing 0.65 kg.).....	0.033	24	0.008
Albino rats (young, 6, weighing 1.02 kg.).....	0.109	32	0.034
Cats (2, weighing 5.25 kg.).....	0.029	23	0.007
Cats (young, 6, weighing 3.8 kg.).....	0.096	43	0.041
Dogs (12, weighing 229.5 kg.).....	0.096	51	0.049
Dogs (young, 4, weighing 14.5 kg.).....	0.085	60	0.051
Man (68 males, goitrous districts).....	0.087	138	0.120
(55 females, goitrous districts).....	0.081	159	0.130
Man (15 males, non-goitrous districts).....	0.086	89	0.077
(26 females, non-goitrous districts).....	0.089	81	0.072

The first series for man have been calculated from an analysis of Baumann's figures for Freiburg, and Oswald's for various parts of Switzerland, the second series from Baumann's figures for specimens from Hamburg and Berlin. The thyroids in these cases were all stated to be normal. In the calculation the assumptions were made that the average weight of man is 70 kgm., of woman, 60 kgm., so that the figures are only approximate. The data for *Squalus sucklii* were calculated from the following measurements:

Sixty-six female fish yielded 1.459 grams dry thyroid tissue. The average weight of ten of these fish, selected at random, was 3.8 kgm.

Sixteen male fish yielded 0.169 gram dry thyroid tissue. Ten of these fish selected at random gave an average weight of 2.5 kgm.

While the averages are derived from too few specimens of each species to permit weight to be attached to the actual figures, they show that there is no parallelism between the iodine content of closely related species, while there does seem to be a fairly close connection between the actual amount of thyroid tissue present. The table further seems to show higher figures for young than for adult animals, both for thyroid, and for the iodine in it. This agrees with the known greater importance of the gland in the growing individual.

Fenger⁸⁸ has shown that iodine is present in the thyroids of the young of sheep, swine, and cattle in amounts of the same order as in the adult animal, and that the same holds true for foetal thyroids in these species. Here placental nourishment furnishes good reasons for a similar distribution of the element between mother and foetus. It will be observed that I have attempted to measure the iodine content of the unborn "pups" of *Squalus sucklii*. From 133 specimens I obtained only 0.06 gram of material (dry weight). This was analyzed, and gave a perfectly negative result, indicating that iodine, if present, was less than 0.01 per cent. In order to be certain that I removed the gland, which was very small, I removed much connective tissue with it, so that the thyroid tissue may not have amounted to more than 50 per cent of the whole material. This would still indicate that the iodine content was less than 0.02 per cent, and therefore bore no near ratio to that in the mother, indicating no transference of nutrient material through the sac during the period of gestation, and probably no storage of iodine in the egg.

Fenger⁸⁹ has also shown that the thyroids of female animals contain higher percentages of iodine. My results for *Scyllium* support this statement, the results for *Squalus sucklii* do not. (The results for male *Squalus sucklii* may be slightly affected by the fact that the tube containing the material in alcohol was broken, and the alcohol lost.) Neither does the analysis of Oswald's and of Baumann's figures for man lend support to the hypothesis. Monery⁹⁰ states that sex has no influence. It may be noted that while Iscovesco⁹¹ claims that in rabbits the relative weights of thyroids in male and female (relative to the whole body weight) are as 5.6 to 7.7, Hatai⁹² states that the thyroid gland does not exhibit any weight difference between the two sexes in the albino rat.

I am unable at present to account for the marked difference in iodine content of the dog-fishes *Squalus* and *Scyllium*. Two explanations suggest themselves. The *Scyllium* material was

⁸⁸ Fenger: this *Journal*, xi, p. 489, 1912; xiv, p. 397, 1913.

⁸⁹ Fenger: *loc. cit.*

⁹⁰ Monery: *Journ. pharm. et chim.*, (6) xix, p. 288, 1904.

⁹¹ Iscovesco: *Cimpt. rend. soc. biol.*, lxxv, p. 252, 1913.

⁹² Hatai: *Amer. Journ. of Anat.*, xv, p. 111, 1913.

obtained in late winter or early spring, the *Squalus* material in late summer. The diet might be richer in iodine at the earlier period. On the other hand, my results for Nanaimo material are distinctly lower than those for material from other places, where comparison is possible (Algae, and Sponges). If the range of individual dogfish is limited, and if the specimens I obtained had not travelled beyond the Straits of Georgia, then the low content may correspond with that in plants and other animals for this region. If neither of these hypotheses based on variations in diet is correct, a specific variation in thyroid tissue, as regards iodine, would appear to be indicated; this seems to me very unlikely for closely related species. That seasonal variations in iodine content of the thyroid do exist has been shown for sheep, cattle, and hogs, by Seidell and Fenger.⁹³ The amount of iodine between June and November is three times as large as that between December and May. Martin's figures⁹⁴ show the same seasonal variations, but to a much less extent. The variation must in these cases be traced to diet.

Parathyroid. I have published in a previous note⁹⁵ a comparison of the iodine content of the parathyroids and thyroids of the dog, and have summarized the evidence which led Estes and Cecil⁹⁶ to the conclusion that the parathyroids do not contain iodine. The iodine content of parathyroids is certainly much smaller than that of the thyroid, indicating differentiation of function as far as the iodine compound is concerned, and also lending some support to the view that iodine is contained in the colloid part of the thyroid gland.

Thymus. Mikulicz tested the therapeutic effect of the thymus tissue in goitre⁹⁷ and claimed to obtain good results with it in eleven cases. Accordingly Baumann tested the calf's thymus for iodine. His first analyses gave negative results,⁹⁸ but subsequently⁹⁹ he obtained positive results to the extent of 0.002 per

⁹³ Seidell and Fenger: this *Journal*, xiii, p. 517, 1913.

⁹⁴ Martin: *Pharmaceut. Journ.*, lxxxix, p. 144, 1912.

⁹⁵ Cameron: this *Journal*, xiii, p. 465, 1913.

⁹⁶ Estes and Cecil: *Johns Hopkins Hosp. Bull.*, xviii, p. 331, 1907.

⁹⁷ Mikulicz: *Berl. klin. Wochenschr.*, xxxii, p. 342, 1895.

⁹⁸ Baumann: *Zeitschr. f. physiol. Chem.*, xxi, p. 319, 1895.

⁹⁹ Baumann: *Münch. med. Wochenschr.*, xliii, p. 309, 1896.

cent. Cunningham¹⁰⁰ states that he has repeatedly tested thymus tissue for iodine with negative results, and suggests that Baumann's positive result was due to the presence of accessory thyroids. Cunningham's conclusion was confirmed by Mendel,¹⁰¹ who, using Baumann's method, found no iodine in the thymuses of children, dogs, or calves. He obtained a trace of iodine in one of three commercial thymus preparations. He concluded that any iodine found present is due to accessory thyroid tissue.

I have thought it desirable to carry out a few further analyses with Hunter's method. My results fully confirm the conclusions of Cunningham and Mendel.

SPECIES	AMOUNT OF THYMUS TAKEN	IODINE FOUND	PER CENT IODINE
Pigeon.....	0.149	0	0.00
Brown Rat.....	0.500	0	0.00
Albino Rat.....	0.114	0	0.00
Guinea pig.....	0.250	0	0.00
Rabbit.....	0.407	0	0.000
Cat (half-grown).....	0.313	0.000006	0.002
Dog.....	0.440	?	doubtful trace
Child.....	0.115	0	0.00
(Glandula Thyroi Merck).....	0.250	0	0.00
	0.500	0	0.000
	0.500	0	0.000
	0.500	0	0.000
	0.500	0	0.000
	0.500	0	0.000

The absence of iodine from the thymus, and also from the parathyroids, does not indicate that these tissues have no function in common with the thyroid. The manifold activities ascribed to the thyroid lead to the inference that its internal secretion probably contains several active substances, one or more of which may contain iodine, others none.¹⁰² If the thymus or parathyroid

¹⁰⁰ Cunningham: *Journ. of Exp. Med.*, iii, p. 231, footnote, 1898.

¹⁰¹ Mendel: *Amer. Journ. of Physiol.*, iii, p. 285, 1900.

¹⁰² Cf. however Biedl: *loc. cit.* In a communication by E. C. Kendall to the N. Y. Society of Experimental Medicine and Biology in May 1913 it was stated that definite proof had been obtained of the presence of more than one entirely active substance in thyroid tissue and that these substances included both iodine and non-iodine compounds. Dr. Kendall has recently informed me that later work has fully confirmed this result.

are able in part to function in place of thyroid tissue, their action must be to produce such thyroid substances as do not contain iodine.

Pituitary. Schnitzler and Ewald¹⁰³ claimed to have found perceptible traces of iodine in two quantities of human pituitaries weighing respectively 19 and 24 grams. They used Baumann's method of analysis.

Wells,¹⁰⁴ using the same method, also obtained positive results.

Halliburton, Chandler, and Sikes, using the same method, found no trace of iodine in 1.29 grams of human pituitaries, nor in 1 gram of ox pituitary (dry tissue).¹⁰⁵ Simpson and Hunter¹⁰⁶ found no trace of iodine in sheep pituitaries, neither under normal conditions, nor after thyroidectomy. Wells,¹⁰⁷ using Baumann's method, found no iodine in the pituitaries from 22 individuals (man), none of whom had received iodine treatment, while 3 pituitaries of iodine patients contained 0.02 milligram.

It may be concluded that under normal conditions the pituitary does not contain iodine. Simpson and Hunter's results do not necessarily indicate no vicarious connection between pituitary and thyroid, but only that the pituitary cannot store up the iodine compound present in the thyroid.

Other Tissues. I have examined the following tissues of the dog.

(1) From a dog whose thyroid (dry weight 0.77 gram) contained 0.176 per cent iodine, employing 0.5 gram of material for each analysis, I obtained perfectly negative results (0.000 per cent) for the following tissues (in each case dried at 100° to constant weight): Heart, lungs, stomach, pancreas, liver, spleen, kidney, intestine, testes, brain, bone, hair, muscle, arteries, veins, tendons, bone-marrow, bladder, blood, salivary glands (mixed sublingual and submaxillary).

(2) From the first dog, and a second (thyroid, dry weight 1.02 grams, 0.074 per cent iodine), the following sampled tissues gave

¹⁰³ Schnitzler and Ewald: *Wiener klin. Wochenschr.*, ix, p. 657, 1896.

¹⁰⁴ Wells: *Journ. Amer. Med. Assoc.*, xxix, p. 1011, 1897.

¹⁰⁵ Halliburton, Chandler and Sikes: *Quart. Journ. of Exp. Physiol.*, ii, p. 229, 1909.

¹⁰⁶ Simpson and Hunter: *ibid.*, iii, p. 121, 1910; iv, p. 257, 1911.

¹⁰⁷ Wells: *this Journal*, vii, p. 259, 1909.

perfectly negative results (0.5 gram dry material for each analysis): prostate, eye-ball, adrenals, lymph glands, sciatic nerve, fat.

(3) From a bitch (thyroid 0.42 gram, iodine content, 0.011 per cent) perfectly negative results were obtained for the ovaries, (0.3 gram), uterus (0.5 gram), and adrenals (0.3 gram).

The tissues of the rabbit were examined with the following results:

(1) Tissues from three male and one female adult rabbits, carefully sampled: Perfectly negative results were obtained, using 0.5 gram material for each analysis, for heart, lungs, stomach, pancreas, liver, kidney, intestine, testes, brain, bone, fur, muscle, bladder, blood, (from heart) submaxillary gland, eye-ball, fat. Doubtful results, which could not be repeated on account of lack of material, were obtained for the adrenals, prostate, ovaries, uterus, and spleen. The mixed thyroid tissue contained 0.064 per cent iodine.

(2) Tissues from two female rabbits were examined as follows: with perfectly negative results, spleen (0.45 gram), ovaries (0.25 gram), uterus (0.5 gram); doubtful result, adrenals (0.45 gram, 0.001 per cent). The thyroid tissue contained 0.137 per cent iodine.

In view of the perfectly negative results obtained with the dog for the prostate and adrenals, these were not further examined in the rabbit.

0.5 gram of Merck's "Suprarenal gland" (cattle or sheep) gave a perfectly negative result.

The tissues of the dog-fish *Squalus sucklii* were examined. The results for the thyroid tissue have been given above (p. 364). The other tissues were taken from two female specimens, except the testes, taken from a male specimen selected at random. In each case 0.5 gram of dried tissue was taken, except where indicated.

Perfectly negative results were obtained for heart (0.1 gram), pancreas, spleen, testes, brain (0.25 gram), bone (vertebrae), skin, muscle, ovaries and eggs, rectal body (0.4 gram). The results for liver and kidney are given in detail:—

DOG-FISH MATERIAL	AMOUNT TAKEN FOR ANALYSIS	IODINE FOUND	PER CENT IODINE
	<i>gram</i>	<i>gram</i>	
Liver oil.....	0.741	0.000000	0.000
Liver residue.....	0.522	0.000015	0.003
	0.533	trace	trace
	0.528	0.000004	0.001
"Dog-fish oil".....	1.500	0.000000	0.000
Kidney.....	0.499	0.000017	0.003
	0.400	0.000012	0.003
			Mean 0.001
			Mean 0.003

A third analysis of the kidney tissue was spoiled, but gave a result of the same order. The liver residue was obtained by heating the liver at 100° for some time, and pouring away the clear oil. It consisted of an oily mass which could not be sampled properly (whence the varying results) and amounting to only three-elevenths of the whole, so that the percentage of iodine in the liver is probably of the order 0.0003 (tissue free from water). The "dog-fish oil" was a sample of the commercial oil sold in Nanaimo, and used for miners' lamps. It was analysed as obtained. Various fish-liver oils have been reported to contain iodine,¹⁰⁸ in amounts varying from 0.0001 to 0.0004 per cent, while "Leberthran" contains greater amounts (according to Stanford,¹⁰⁹ cod liver oil dragées contain 0.056 per cent). The "residue" I examined is probably not comparable, since it would have yielded much more oil at a higher temperature.

It is to be observed that the tissues of the dog-fish for which positive results were obtained were those of excretory organs.

I have elsewhere summarised the evidence showing that fish-tissue usually contains iodine.¹¹⁰ Bourcet's results (from an examination of 25 species) indicate that the limits for sea-fish are 0.000007 (*Leuciscus cephalus*) and 0.00024 per cent (*Merlangus carbonarius*).¹¹¹

Doyon and Chenu¹¹² have published a few results for reptilian tissue. They have observed that the carapace of the African

¹⁰⁸ Cf. for example, Stanford: *Chemical News*, xlviii, p. 233, 1883.

¹⁰⁹ Stanford: *loc. cit.*

¹¹⁰ Cameron: *Biochem. Journ.*, vii, p. 466, 1913.

¹¹¹ Bourcet: *Compt. rend. de l'Acad. des Sci.*, cxxviii, p. 1120, 1899.

¹¹² Doyon and Chenu: *ibid.*, cxxxix, p. 157, 1904.

tortoise (fresh tissue) contains 0.00006 per cent, situated chiefly in the shell and not in the bony part. They found also that the eggs contained a trace of iodine.

The following data are available for mammalian tissue:

Barell¹¹³ found (Bn) traces of iodine in the ovaries of swine and cattle (0.0006 per cent), in adrenal tissue (0.0003 per cent), in spleen (0.0015 to 0.002 per cent), and in various commercial preparations of these glands.

Cunningham twice found traces of iodine in the salivary gland of the ox, and once in the parotid gland of the dog (Bn).¹¹⁴

Gley found 0.0001 per cent iodine in a dog's spleen (dry tissue) and 0.000038 per cent in a rabbit's liver, but obtained no measurable traces in adrenals, lymphatic ganglia, thymus, pituitary, or ovary of the rabbit (method Bt).¹¹⁵

Gley and Bourcet¹¹⁶ found that dog's blood contained very variable amounts of iodine (limits 0.013 to 0.06 mgm. per liter). Gley¹¹⁷ at first stated that the iodine is present in the red blood-corpuscles, and only in the blood of adults; it is absent from the blood of new-born animals. Gley and Bourcet later concluded that the iodine is present in the serum in protein combination.

Howald¹¹⁸ (method Bn) found that normal human hair and fowl's feathers contain no iodine; that it is excreted in small part through the hair, when administered *per os*; and that it occurs in the hair of thyroidectomized animals.

Mendel¹¹⁹ found no iodine in the ovarian substance of swine, nor in the salivary gland of the ox (method Bn).

Justus' results I have already referred to (p. 339).

Neu¹²⁰ reported that 60 per cent of ovarian tissue examined (human) contained iodine, the average amount being 0.64 mgm. per gram fresh substance. Uterus and myoma tissue gave negative results (method Bn).

¹¹³ Barell: *Pharm. Ztg.*, xlii, p. 130, 1897.

¹¹⁴ Cunningham: *loc. cit.*

¹¹⁵ Gley: *Rev. gén. sciences pures et appl.*, ix, p. 13.

¹¹⁶ Gley and Bourcet: *Compt. rend. de l'Acad. des Sci.*, cxxx, p. 1721, 1900.

¹¹⁷ Gley: *La semaine med.*, xviii, p. 237, 1898.

¹¹⁸ Howald: *Zeitschr. f. physiol. Chem.*, xxiii, p. 209, 1897.

¹¹⁹ Mendel: *loc. cit.*

¹²⁰ Neu: *Munch. med. Wochenschr.*, lix, p. 73, 1912.

Zoeppritz¹²¹ found no trace of iodine in 9.1 grams ovaries, 4.25 grams ovaries, 5.1 grams vagina, 5.59 grams uterus (fresh tissue, swine). Five human ovaries gave perfectly negative results (3 from operations, 2 from corpses). In several cases the ovaries of women were examined, to whom iodide or "Lipoiden" tabloids had been given, previous to operation. These also yielded negative results. Zoeppritz used Baumann's method, and from test experiments concluded that his negative results indicated that less than 0.15 mgm. iodine was present in each experiment.

Iodine has been reported present in hen's eggs by Bonanni.¹²² He states that eggs laid in the country in May-June had a higher iodine content than those laid in the city in February-March. I have been unable to see his actual figures.

The series of results just quoted are either contradictory, or refer to amount of iodine less than 0.001 per cent.

The most accurate analyses of mammalian tissue so far published are undoubtedly those of Bourcet,¹²³ using his own method. His figures lead to the following data for rabbits (3 males examined, weighing together 4.2 kgm. The figures presumably refer to fresh tissue).

Blood, 0.0000025; muscle (heart), 0.00001; large intestine and contents, 0.0000025; small intestine and contents, 0.00001; bladder, 0.00000, stomach, 0.000008; liver and bile duct, 0.00017; kidney, 0.00003; fat, 0.000000; hair, 0.0018; muscle, 0.000005; lungs, 0.00007; genital apparatus, 0.00006; brain, 0.00004; pancreas, 0.00000; skin (without fur), 0.0006; eye-ball, 0.00000.

He obtained similar results for a dog, weighing 8 kilograms. Positive results were obtained in this case also for the thymus, pituitary, and medullary tissue, for the mammary gland, and the gravid uterus. He considers that all the glands of the body contain iodine, though in much less amount than the thyroid. The iodine is excreted chiefly by the skin, under normal conditions, since while the faeces and urine contain very little iodine, and the saliva also, the sweat, skin, hair, and nails, contain relatively large amounts. The hair is the chief agent of excretion, containing

¹²¹ Zoeppritz: *ibid.*, lix, p. 1898, 1912.

¹²² Bonanni: *Boll. r. accad. med. Roma.*, xxxviii, through *Chem. Abstr.*, vii, p. 2961, 1913.

¹²³ Bourcet: *Comp. rend. de l'Acad. des. Sci.*, cxxxi, p. 392, 1900.

on the average 2.5 mgm. per kilogram. In women menstrual blood contains more iodine than normal blood. Five cases showed figures varying between 0.8 and 0.9 mgm. per kgm., while in a sixth direct comparison gave the respective figures 0.94 and 0.021 mgm. per kgm.

Remembering that the usual ratio of fresh to dry tissue varies from 3:1 to 5:1, Bourcet's figures are not in disagreement with my results, except perhaps in the case of hair or fur. It is doubtful whether, in drawing his conclusion as to the method of excretion, he has laid sufficient stress on the relative extent to which excretion takes place through the skin, and through the kidneys. My results with the dog-fish certainly seem to indicate that in fishes excretion through the kidney is the chief method of elimination.

DISCUSSION OF RESULTS

From the data given in the previous section a number of conclusions can be drawn with tolerable certainty. Iodine appears to be an invariable constituent of all marine Algae, in amounts greater than 0.001 per cent. There does not appear to be any specific difference between the amounts present in Brown and in Red Algae, since, while the *Laminaria* undoubtedly show a higher average than other Brown Algae, certain Red Algae (*Nitophyllum*), growing under similar conditions, give figures of the same order. The results for Green Algae are too few to permit of a similar generalization. Distinct variation of iodine content can occur in the same species, growing under almost the same conditions, and in different, but closely related species. No cause can yet be assigned with certainty to explain such differences. On the other hand, the marked difference between fresh-water plants and vegetables, on the one hand, and marine Algae on the other is due to difference in the iodine content of the environment, and therefore in the diet of these plants. The difference in content in different species of vegetables (Bourcet) parallels that for different species of Algae growing under similar conditions, and suggests a specific quantitative action of the plant cell in retaining iodine.

Iodine is present in appreciable quantity in certain tissues of all marine species. As we get higher in the scale there is more differentiation (and probably less total iodine in the whole organism)

until in vertebrates thyroid tissue alone is of consequence. Bourcet¹²⁴ has traced out the general biological cycle of iodine. The cycle in marine organisms is fairly clear. There is evidently a continuous circulation of the element in a succession of living organisms, starting with Algae (and especially Diatoms). Death and subsequent decay of a certain proportion of animals and plants returns organic and inorganic iodine to the sea-water itself, whence it is again removed by Algae. Gautier's results for the iodine content of sea-water, if confirmed, and applied rigorously, would indicate that the Algae themselves obtain their iodine in organic form. This is perhaps not absolutely impossible, since various authors seem to have shown that Algae can assimilate organic material, including amino-acids¹²⁵ (and, as mentioned in the introduction, it has been shown beyond doubt that at least part of the iodine in organic combination is present in amino-acid groups), but without further data it seems more probable that a minimal quantity of iodine reaches the inorganic stage, and is then reabsorbed by the Algae, and the cycle completed.

(Very little work appears to have been carried out to determine the form in which iodine occurs in Algae. Eschle¹²⁶ showed that in *Fucus vesiculosus* and *Laminaria digitata* the iodine was present almost completely in organic combination, and considered that several different organic compounds containing iodine were present. Oswald¹²⁷ has also investigated *Fucus vesiculosus*, but has obtained no definite result.)

Of mammalian tissue, the thyroid alone is of importance in connection with the storage of iodine. It is to be expected that where there is a circulation of iodine in the organism, other tissues will also contain minute amounts. (This is supported by results of many observers, indicating specific retaining power by different tissues when iodine compounds are injected into the blood, or administered in large amounts *per os*.) Bourcet's results indicate that this is indeed the case, but the smallness of his figures (confirmed by my own negative results with a less rigorous method) indicates that the presence of iodine in such

¹²⁴ Bourcet: *ibid.*, cxxxii, p. 1364, 1901.

¹²⁵ See Oltmann's *Morphologie u. Biologie der Algen*, 1905, ii, p. 155.

¹²⁶ Eschle: *Zeitschr. f. physiol. Chem.*, xxiii, p. 30, 1897.

¹²⁷ Oswald: *ibid.*, lxxv, p. 353, 1911.

tissues is in all probability without significance. It is less than 0.001 per cent in all non-thyroid tissue.

In thyroid tissue marked variations of iodine content occur, both in individuals of the same species, and in different species. Such variations are all traceable to differences in diet.

I have already summarized the evidence bearing on the relation of sex to iodine content.

Iodine is an invariable constituent of normal thyroid tissue, and under normal conditions the diet always contains sufficient iodine for the upkeep of a minimal amount. The minimal quantity appears to be of the order 0.01 per cent, the maximal quantity so far observed being 1.16 per cent.

Three different tissues of marine animals, reported on in this paper, contain iodine in marked quantity, the test of the tunicate *Pyura*, the outer cuticle of the horse-clam *Schizothoërus*, and the inner tube of the worm *Diopatra*. The test of the tunicate is stated to consist largely of cellulose,¹²⁸ while the organic material of the tube of another Eunicid worm (*Hyalinoecia*) is stated to consist in part of *onuphin*, a compound which, although containing nitrogen in small quantity, seems closely related to dextrin or glycogen.¹²⁹ On the other hand the cuticle of the horse-clam is almost certainly of a keratin nature, so corresponding to the sclero-proteins found in Sponges (spongin) and Corals (gorgonin).

From examination of various iodo-proteins (both those occurring in nature, and others prepared in the laboratory) the iodine linkage has so far been located in certain amino-acids only; in tyrosine with certainty, as 3,5-di-iodo-1-tyrosine;¹³⁰ and possibly also in tryptophane¹³¹ (as mono- or di-iodo-tryptophane), and phenylalanine¹³² (as paraiodophenylalanine). There is so far no evidence of any other type of combination. Such other type

¹²⁸ Cf. Winterstein: *ibid.*, xviii, p. 43, 1894.

¹²⁹ Schmiedeberg: *Mitt. a. d. zool. Station zu Neapel*, iii, p. 373, 1882.

¹³⁰ See for example, Wheeler and Jamieson: *Amer. Chem. Journ.*, xxxvi, p. 365, 1905; Wheeler and Johns: *ibid.*, xlv, p. 11, 1910; Wheeler and Mendel: this *Journal*, vii, p. 1, 1909; Oswald: *Zeitschr. f. physiol. Chem.*, lxxv, p. 353, 1911; etc.

¹³¹ See for example, Nürnberg: *Beitr. f. chem. Physiol. u. Path.*, x, p. 125, 1907; Biochem. *Zeitschr.*, xvi, p. 87, 1909; Neuberg: *ibid.*, vi, p. 276, 1907; Oswald: *Arch. f. exp. Path. u. Pharm.*, ix, p. 115, 1908.

¹³² See Wheeler and Clapp: *Amer. Chem. Journ.*, xl, p. 458, 1908.

is suggested by the results for the Ascidian test and the Worm tube, and I hope to examine this material further.

I wish to acknowledge my grateful indebtedness to Dr. Maclean Fraser, the Curator of the Biological Station at Nanaimo, B. C., for his uniform kindness in assisting me in the work of collection and identification of much of the material used in this paper, to thank Mr. F. S. Collins and Mr. A. Klugh for kindly identifying a number of Algae for me, and to thank Professors Swale Vincent and Buller for their interest and encouragement in the course of this work.

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The work forms part of researches carried out under the direction of the Ductless Glands Committee of the British Association

THE EFFECT OF ACUTE DESTRUCTIVE LESIONS OF THE LIVER ON ITS EFFICIENCY IN THE REDUCTION OF THE AMMONIA CONTENT OF THE BLOOD.¹

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(Received for publication, June 4, 1914.)

In view of the many attempts that have been made to devise clinical methods for the determination of the functional capacity of the liver, it is important to learn in what degree experimental lesions may alter the power of the liver to perform its multiple functions. The present study has to deal with that function of the liver, the evidence of which lies in the fact that blood after passing through the liver becomes poorer in ammonia content. In a previous publication² it was shown that perfusing fluids to which was added ammonium carbonate (14–28 mgm. ammonia N per 100 cc.) after being repeatedly passed through the normal liver for from one-half to one hour showed a reduction of ammonia to from 4 to 7 mgm. per 100 cc. It was with this result in view that essentially the same method was applied to pathological livers. Folin and Denis³ have shown that the ammonia of the portal blood is chiefly of putrefactive origin; whether or not the liver is the sole means of protection of the organism against this and other toxic products of putrefaction is not at issue here, but that the liver serves some such function cannot be doubted. There is little doubt that in human cases of cirrhosis of the liver and other more destructive lesions this function of the liver is not seriously interfered with for although cases show an excess of ammonia in the urine, this is probably due to acidosis; Muenzer⁴ showed that the administration of alkali reduced the urinary ammonia to normal.

¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² Fiske and Karsner: this *Journal*, xvi, p. 599, 1913.

³ This *Journal*, xi, p. 161, 1912.

⁴ *Deutsch. Arch. f. klin. Med.*, xlv, p. 429, 1889.

It is of importance, however, to know if there is any interference with this function in experimental lesions when larger areas of liver substance are destroyed than is the case at least in human cirrhosis. It is probable a priori that the factor of safety in the liver is so great that no such alteration of function occurs, at least when the amount of destruction is compatible with life, and the results of the experiments are in accordance with this assumption.

For the purposes of this study normal and pathological livers of cats were perfused with about 80 cc. of defibrinated blood of normal cats to which was added about 20 cc. of Ringer's solution containing a measured amount of ammonium carbonate. In the case of the normal animals, their own blood was used; in the case of the pathological animals, normal cats were bled immediately before the perfusion so that fresh normal blood was obtained for each experiment. The technique was the same as that used in the previous study except that on each passage of fluid into the bottles there was simultaneous introduction of pure oxygen (oxone generator); the receiving bottle was vigorously shaken so that when the blood reentered the liver, it was bright red. Samples of the perfusing fluid were taken immediately before perfusion and immediately after perfusion, the process lasting for one hour. The determination of ammonia was by the same method as in the previous study.⁵ Ammonia was added in the form of a standard aqueous solution of commercial ammonium carbonate. The solution, therefore, contained in addition to the neutral salt also ammonium bicarbonate and ammonium carbamate. The removal of the nitrogen of the last named salt by the air current requires a longer time than is the case with ordinary ammonium salts; there is undoubtedly shifting of equilibrium towards the carbonate side as the concentration of NH_4^+ is reduced by aeration. In the case of blood containing the commercial salt in concentration of about 30 mgm. per 100 cc. expressed as N, as will be seen from the following table it will be found necessary to take 1.0 cc. fluid and blow over for one hour in order to recover all the added ammonia N. The figures given for ammonia N include also that existing as carbamate.

⁵ Fiske and Karsner; *loc. cit.*

TABLE I.

EXPERIMENT NUMBER	VOLUME OF FLUID	TIME BLOWN	AMMONIA N ADDED	AMMONIA N RECOVERED
	cc.	minutes	mgm.	mgm.
1	5.0	30	32.8	19.8
2	5.0	60	32.8	22.4
3	1.0	30	32.8	25.8
4	1.0	30	32.8	27.8
5	1.0	60	32.8	32.4
6	1.0	60	32.8	31.5
7	1.0	60	16.4	15.9

In the experiments to be tabulated later, duplicate determinations were made on samples of 1.0 cc. taken before perfusion and blown for one hour. After perfusion the original procedure of Folin and Denis was employed, the small amount of ammonia N present being completely removed from 5 to 10 cc. of fluid in twenty to thirty minutes.

In the experiments reported previously with normal livers and one case of obstructive cirrhosis the fluid at the end of one hour's perfusion (one-half hour in the case of the cirrhotic liver) contained 5 to 7 mgm. ammonia nitrogen per 100 cc. as compared with 21 to 28 mgm. at the beginning. The use of pure oxygen and thorough shaking for purposes of oxygenation reduced this residual ammonia to less than 0.5 mgm. per 100 c.c., which under the conditions of the experiment is fairly close to the normal amount of blood (about 0.1 mgm.). One reason for this difference is probably the accumulation of lactic acid in the fluid when oxygenation is less complete, ammonia being retained for neutralization.

The following agents were used for the production of the destructive lesions of the liver: chloroform, specific hemolytic immune serum, phosphorus and hydrazine. A cat was also given repeated injections of phlorhizin according to Lusk's method. The work was controlled by histologic sections stained for fat and by the hematoxylin-eosin method. The chloroform liver showed necrosis (partly hyalin) of the liver lobule through about two-thirds of its diameter. The immune serum liver showed extensive hyalin necrosis of very irregular distribution, but very little thrombosis, the thrombi probably being washed out by the perfusion. Both the phos-

phorus and hydrazine livers showed fatty transformation of practically every parenchymal cell.

The following table gives the experiments in the order in which they were performed.

TABLE II.

CAT NUMBER	WEIGHT KILOS	VOLUME OF FLUID		AMMONIA NITROGEN MGM. PER 100 CC.		REMARKS
		In	Out	Before	After	
7	1.5	96	129	33	0.33	Normal.
9	1.8	108	131	29	0.36	Chloroform <i>per os</i> . 1.5 cc. per kilo on first day. 0.75 cc. per kilo on second day. Perfused 24 hrs. after last dose.
12	2.1	126	160	31	0.46	Phosphorus (suspended in olive oil) <i>per os</i> . 11 mgm. per kilo first day. 18 mgm. per kilo second day. 15 mgm. per kilo third day. Perfused 24 hrs. after last dose.
16	1.5	83	106	17	0.39	Phlorhizin subcutaneously. Starved 2 days, followed by 1.0 gm. phlorhizin (Merek) in 10 cc. 1% Na_2CO_3 three times daily for 2 days. Perfused third day. G:N = 2.6.
17	2.6	96	121	31	0.14	Hemolytic immune serum (rabbit) 0.5 cc. (50 amboceptor unit) intravenously 24 hrs. before perfusion.
18	2.9	108	139	28	0.17	Hydrazine sulphate. 100 mgm. per kilo subcutaneously 48 hours before perfusion (aqueous solution).
20	3.2	76	95	38	0.10	Normal.

It will be seen in this table that the reduction in ammonia content as the result of one hour's perfusion is very marked and it will be seen further that as the manipulation of the device for oxygenation became more skillful, the reduction of ammonia content was greater. This difference after perfusion is of no significance, however, in the final interpretation of results, the important point being the actual decrease during the perfusion. The small amounts of ammonia remaining may be expected to vary

with the completeness of oxygenation and doubtless with other unknown factors. The fact that the ammonia does not disappear completely is perhaps dependent upon the existence of a physico-chemical equilibrium between the ammonia and some other constituent or constituents of the fluid. The possibility that such an equilibrium exists between urea and ammonia has at present no great support on biochemical grounds,⁶ but from the standpoint of physical chemistry it seems quite probable.

CONCLUSIONS.

1. Oxygenation of the perfusing fluid by pure oxygen instead of air renders more complete the reduction of the ammonia content of the fluid as it passes through the liver.

2. There is no difference between normal livers and livers poisoned with the various toxic substances mentioned with respect to their ability to lower the ammonia content of blood perfused through them.

⁶ Wakeman and Dakin: this *Journal*, ix, p. 327, 1911.

STUDIES IN THE BIOCHEMISTRY OF PURINE METABOLISM.

I. THE EXCRETION OF PURINE CATABOLITES IN THE URINE OF MARSUPIALS, RODENTS AND CARNIVORA.

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(Received for publication, June 10, 1914.)

In preliminary communications from this laboratory¹ there have been reported figures showing the relative proportions in which the known products of purine catabolism—allantoin, uric acid and purine bases—may be expected to appear in the urine of a variety of mammalian species. It is proposed to present in this and a subsequent paper a detailed account of the observations on which these figures were based.² The point of view from which the investigation was undertaken has been explained in a previous publication.³ Briefly, it was hoped that a survey, on the plan indicated, of all the species immediately available might reveal the existence of several types of purine metabolism, linking the extremes exemplified by man and dog, and reflecting perhaps more or less closely the phylogenetic relationships of the different orders.

The general method adopted may be described very shortly.

One or more specimens of the species under observation were confined in an appropriate metabolism cage. Food was then limited to materials as nearly as possible free from allantoin or purines, or, in some instances, was

¹ Givens and Hunter: this *Journal*, xiv, Proc. Soc. Biol. Chem., p. xxiv, 1913; Hunter and Givens: *ibid.*, xvii, Proc. Soc. Biol. Chem., p. xxiii, 1914.

² The unimportant differences that exist here and there between the figures of our earlier reports and the averages given in the present paper result simply from the inclusion of further experimental material.

³ Hunter and Givens: this *Journal*, xiii, p. 371, 1912.

entirely withheld. The urine voided was collected at suitable equal intervals, made up with cage washings to a definite volume, and immediately analyzed. When necessary, care was taken, by charging the receptacle with acetic acid, to counteract any natural alkalinity of the urine, and liberal use was made of thymol as an antiseptic. When the animal was a reasonably large one, the daily excretion of a single specimen was made use of. Otherwise the period of collection was extended, or a number of individuals was kept in one cage, and the analyses made upon the mixed urine of the group. Such a procedure has obvious drawbacks, but unfortunately, while uric acid may now be determined with a very small amount of urine, the estimation of allantoin, and still more of purine bases, calls for considerable quantities.

Analytical Methods. Total nitrogen was determined by the method of Kjeldahl. Uric acid and purine bases were separated according to the procedure of Krüger-Schmid; the former was determined sometimes by estimation of nitrogen (Kjeldahl) in the isolated crystals, sometimes by applying the Folin-Macallum color reaction⁴ to an aliquot part of the purine solution. In one or two more recent analyses uric acid was determined by applying the Folin-Denis⁵ method directly to the urine. Allantoin was estimated by the method of Wiechowski,⁶ in the application of which, while permitting ourselves every liberty in modifying according to circumstances the preliminary treatment of the urine, we adhered strictly, in the final precipitation, to the conditions laid down by Wiechowski as essential to a quantitative result; nearly all the figures as reported are calculated from the nitrogen content of the mercury precipitate, but for every separate series of urines we satisfied ourselves by actual isolation and analysis of allantoin in substance, that the mercury precipitate contained no appreciable amount of any other nitrogenous substance.⁷

In reviewing our results we have found it convenient to introduce terms that concisely indicate a few of the more frequently mentioned ratios. Thus, of the total urinary nitrogen of purine derivation a certain fraction, more or less characteristic for each species, appears as allantoin; that fraction, expressed as a percentage, we have called the "*allantoin ratio*." "*Uricolytic index*" means the ratio of allantoin nitrogen to the sum (again taken as 100) of allantoin and uric acid nitrogen only; it is taken as the measure of an animal's

⁴ Folin and Macallum: this *Journal*, xi, p. 265, 1912; xiii, p. 363, 1912.

⁵ Folin and Denis: this *Journal*, xiv, p. 95, 1913.

⁶ Wiechowski: *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 109, 1908.

⁷ It is true that the isolated allantoin was not always pure until recrystallized. But the impure material contained always less, never more, nitrogen than the theoretical amount. The impurities were therefore poor in nitrogen, and must have introduced an error practically negligible.

capacity to oxidize uric acid arising intermediately.⁸ The number of milligrams of endogenous purine-allantoin nitrogen excreted daily per kilogram of body weight we propose to call the "*purine coefficient*;" this is regarded as an expression of the relative intensity of purine metabolism as a whole.

The present paper deals with observations upon six species, representative of three mammalian orders: (I) *Carnivora* (badger, coyote, raccoon); (II) *rodentia* (guinea-pig, rat); (III) *marsupialia* (opossum). In discussing these we shall utilize for comparison data furnished by other workers for the dog and the rabbit, so that altogether eight species will be considered. Since the material available as a standard is most abundant in the case of the dog, it will be convenient to present first the results with carnivores.

I. ORDER CARNIVORA.

1. The Badger (*Taxidia taxus*)—Family *Mustelidae*.⁹

Two badgers, one (male) weighing 4.4, the other (female) 4.7 kgm., were kept in one metabolism cage and fed on bread and water (*Experiment 1*). Urine was collected quantitatively at the same hour daily. Since the animals took a great deal of water, and micturated with considerable regularity soon after drinking, it was possible to arrange that each collection represented very nearly a twenty-four hour specimen. Five such specimens were collected on five consecutive days. In addition we analyzed a day's sample of urine obtained during a period of starvation (*Experiment 2*). The urine was always acid. The allantoin determinations were made according to the latest scheme of Wiechowski.¹⁰ The allantoin, when isolated, was, as usual with carnivore urines, clean, well crystallized and all but absolutely pure. For the determination of the purines we followed the scheme already employed in this

⁸ Wiechowski (*Biochem. Zeitschr.*, xix, p. 368, 1909) expresses the same relation in terms, not of nitrogen, but of "uric acid decomposed." Since uric acid and allantoin contain nearly the same proportion of nitrogen, the numbers given by either method of reckoning are all but identical.

⁹ We have followed in the main the classification and nomenclature adopted by Miller in *North American Mammals*, Bulletin 79, U.S. National Museum.

¹⁰ Ellinger's *Analyse des Harns*, ii, p. 1076, Wiesbaden 1913.

laboratory for analysis of monkey urine.¹¹ The results are to be found in Table I.

TABLE I.
The Badger.

EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEIGHT OF ANIMALS	DAYS OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric acid	Bases	Sum	Per cent of total N	Allantoin	Uric acid	Bases		
		kgms.		grams	mgms.	mgms.	mgms.	mgms.						
1	2	9.1	1	3.38	262	5.73	3.8	271.5	8.0	96.5	2.1	1.4	98	30
			2	3.34	263	5.52	2.9	271.4	8.1	96.9	2.0	1.1	98	30
			3	3.01	235	4.72	2.4	242.1	8.0	97.0	1.9	1.0	98	27
			4	3.01	250	4.43	3.5	257.9	8.6	96.9	1.7	1.4	98	28
			5	2.96	224	4.42	2.3	230.7	7.8	97.0	1.9	1.0	98	25
2	2	9.0	1	2.85	241	4.03	3.0	248.0	8.7	97.2	1.6	1.2	98	27
Av.									8.2	96.9	1.9	1.2	98	28

Discussion. The natural standard with which to compare a carnivore is of course the dog. In the appended Table II we have collected from the literature some data in regard to the purine metabolism of that animal. All the figures refer to twenty-four hour periods.

A comparison of Table I and II shows that in every characteristic the purine metabolism of the badger is identical with that of the dog. The correspondence affects not only the distribution of the allantoin-purine nitrogen, but even, it may be pointed out, its ratio to the total nitrogen excretion. The only relation which shows any irregularity at all is the purine coefficient. Even this is of entirely the same order of magnitude, so long as we confine comparison to badgers and dogs of approximately equal size. Only in the case of the very large dog is it decidedly smaller. This is a point of some interest, since it appears to indicate that, even within the bounds of the same species, the intensity of purine catabolism is to some extent dependent upon size.

In both dog and badger the capacity for uric acid oxidation is so great that the uricolytic index approaches 100, and the amount of

¹¹ Hunter and Givens: this *Journal*, xvii, p. 37, 1914.

TABLE II.

The Dog.

WEIGHT OF ANIMAL	TOTAL NITROGEN	PURINE-ALLANTOIN N		PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT	DIET	AUTHORITY
		Total	In per cent of total N	Allantoin	Uric acid	Bases				
kgms.	grams	mgms.								
3.5	1.07	117.8	11.0	96.8	1.8	1.4	98	33	Mixed	Schittenhelm*
4.7	1.64	159	9.7	98.5	+1.5		98(?)	34	"	Hirskawa†
4.8	1.62	138	8.5	97.9	1.7	0.4	98	29	"	"
4.9	1.57	114.9	7.3	96.6	1.8	1.6	98	31	None	Schittenhelm*
5.2	1.92	160	8.3	96.2	2.5	1.3	97	31	Mixed	"
18.0	3.82	303	7.9	96.4	1.9	1.7	98	17	"	"
Av.			8.8	97.1	1.9	1.3	98	29		

* *Zeitschr. f. Physiol. Chem.*, lxii, p. 80, 1909† *Biochem. Zeitschr.*, xxvi, p. 441, 1910

uric acid excreted is almost a negligible quantity. The purine bases of the urine are generally even more insignificant in amount. The catabolism of purines may for practical purposes be measured by the allantoin output alone, and this may, under ordinary conditions of diet or even in starvation, be so great as to account for nearly one-tenth of all the nitrogen excreted. We have here then a well-defined type of purine metabolism, exemplified by at least two families (*Canidae* and *Mustelidae*) of the carnivora. This type is further illustrated by the coyote.

2. The Coyote (*Canis latrans*)—Family *Canidae*.

An earlier communication from this laboratory,¹² dealing with the nitrogen metabolism of the coyote, contains some data that have a direct bearing on the present subject. It would be superfluous to reproduce here the analytical figures already published. It is sufficient to say that during an eight-day starvation period a coyote, weighing 6.7 kgm., was found to excrete an average of 145 mgm. of allantoin nitrogen and 6.7 mgm. of purine nitrogen daily. The purine nitrogen includes both uric acid and bases, the determination of each separately being at that time impossible

¹² Hunter and Givens: this *Journal*, viii, p. 449, 1910.

with the small amount of urine available. The figures indicate for the coyote a purine coefficient of 23, and an allantoin ratio of 95.6. Making the reasonable assumption that the purine nitrogen was divided between uric acid and bases in much the same proportion as with the dog, we can estimate the output of uric acid nitrogen as about 4 mgm. This would give a uricolytic index of 97. The endogenous purine metabolism of the coyote is therefore in all respects of the same character as that of the dog and the badger.

3. The Raccoon (*Procyon lotor*)—Family *Procyonidae*.

According to Seton¹³ the full-grown male raccoon weighs from 18 to 22 pounds. Compared with this standard the three specimens which we secured were undersized. The first, said to be about three years old, weighed only 2.8 kgm. It was kept in a cage by itself, and its urine collected in 48-hour periods (*Experiment 3*). Since this animal had the habit of micturating whenever its cage was washed out, the periods could be very sharply separated. The other two animals were only one year of age, and weighed together 5.3 kgm; from them a single day's urine was obtained (*Experiment 4*). The diet in each case consisted of bread, peanuts and hard-boiled eggs. The nuts were fed separately. The eggs with their shells were thoroughly incorporated with the bread by kneading with a little water. The admixture of egg shell had the effect of rendering the feces firm. Drinking water was furnished in a cup with a very small mouth, so that the raccoons were unable to follow their habit of washing food before eating. The allantoin determinations were made by the earliest Wiechowski method.¹⁴ The substance was readily obtained in large, clear, apparently clean crystals, which however contained a small proportion of non-nitrogenous material. The purines were determined as with the badger, by following the scheme first employed for monkey urine.

Table III contains the results with the raccoon.

Discussion. In general the picture presented by the table resembles that given by the other carnivores. The single raccoon of *Experiment 3* does, however, seem to excrete a rather high pro-

¹³ Seton: *The Life History of Northern Animals*, ii, p. 1010, New York, 1909.

¹⁴ Wiechowski: *loc. cit.*, 1908.

TABLE III.

The Raccoon.

EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEIGHT OF ANIMALS	DAYS OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric acid	Bases	Sum	Per cent of total N	Allantoin	Uric acid	Bases		
		kgms.		grams	mgms.	mgms.	mgms.	mgms.						
3	1	2.8	1-2	1.89*	50.6	4.0	1.3	55.9	3.0	90.6	7.2	2.3	93	—
			3-4	2.87	85.5	5.7	1.9	93.1	3.2	91.8	6.1	2.0	94	17
			5-6	3.68	84.2	5.2	1.9	91.3	2.5	92.3	5.7	2.1	94	16
4	2	5.3	1	2.33	83.5	2.2	1.6	87.3	3.7	95.7	2.5	1.8	97	16
Av.									3.1	92.6	5.4	2.0	95	16

* A considerable portion of this period's urine was lost.

portion of uric acid, so that the uricolytic index is not quite so near to 100 as usual. The ratios for this specimen in fact rather resemble those of the rabbit and guinea-pig (see later) than those of most dogs. It is impossible as yet to say from the material at hand, whether such slight differences have any significance. On the one hand it seems that dogs sometimes excrete relatively more uric acid than appears in the results quoted in Table II;¹⁵ and on the other the raccoons of *Experiment 4* showed a uricolytic index quite up to that of most dogs. A more definite distinction between the raccoon and the other carnivora studied may be noted in the comparatively low purine coefficient of the former. Assuming that this is not a mere individual peculiarity of the particular raccoons in our possession, it would be interesting to ascertain whether it is a characteristic of the species only, or of the whole group of *Procyonidae* to which it belongs. It is quite conceivable that within the entire order of carnivora there may be discovered special family characteristics of more or less pronounced degree.¹⁶

¹⁵ To take a random instance, the figures reported by Wiechowski (*loc. cit.*, 1903) for his Dog B give a uricolytic index of 94.

¹⁶ To the list of carnivora in which the allantoin and uric acid output have been compared there should be added the cat. Wiechowski (*loc. cit.*, 1903) found in 60 cc. of cat's urine 90 milligrams of allantoin and 3 of uric acid. This gives a uricolytic index of 97.

II. ORDER RODENTIA

1. The Guinea-Pig (*Cavia*)—Family *Caviidae*.

The urine of the guinea-pig living upon its natural vegetarian diet is an exceedingly turbid, highly pigmented, alkaline fluid, which filters with extreme difficulty. It proved to be not quite a simple matter to isolate allantoin from it in a quantitative way. The most successful technique, and the one which gave the largest yields was that which we have applied to the urine of the monkey.¹⁷ This technique was employed in the series of determinations reported below. Other series, in which the proportion of allantoin found was somewhat smaller (the uricolytic index occasionally appeared to be as low as 80), have been discarded as probably inaccurate. For the purine determinations it was found advantageous to follow the suggestion of Schittenhelm,¹⁸ and subject the urine to a preliminary hydrolysis with 3 per cent sulphuric acid. The decomposition of the copper precipitate was best carried out by hydrogen sulphide in a boiling acid solution.

In *Experiment 5* nine guinea-pigs, weighing altogether 3.9 kgm., were maintained upon a diet of cabbage leaves, the urine being collected and analyzed daily. On the fourth day four of the animals were withdrawn, the five left weighing 2.3 kgm.; this forms *Experiment 6*. For *Experiment 7* six other guinea-pigs, total weight 2.8 kgm., were fed cabbage, lettuce, hay and carrots, while the urine was collected in four day periods; here the whole excretion, except that required for the total nitrogen determination, was utilized for the more precise determination of the relation between uric acid and bases. The results of the experiments are exhibited in Table IV.

Discussion. It is clear from the table that the guinea-pig possesses in a very high degree the power of oxidizing uric acid. Its uricolytic index of 94 approaches very closely that of the Canidae. In spite of this the amount of nitrogen eliminated as uric acid is by no means inconsiderable. From the data given it may be calculated to lie between 1.3 and 2.5 mgm. per kilogram of body weight daily, with an average of 1.7. This is not much below the per

¹⁷ Hunter and Givens: *loc. cit.*, 1912.

¹⁸ Aberhalden's *Handbuch der biochemischen Arbeitsmethoden*, iii, p. 887, Berlin, 1910.

TABLE IV.
The Guinea-Pig.

EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEIGHT OF ANIMALS	DAYS OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric acid	Bases	Sum	Per cent of total N	Allantoin	Uric acid	Bases		
		kgms.		grams	mgms.	mgms.	mgms.	mgms.						
5	9	3.9	1	2.97	96	5.1	3.3	104.4	3.5	91.9	4.9	3.2	95	27
			2	3.09	89	5.0	3.8	97.8	3.2	91.0	5.1	3.9	95	25
			3	2.34	111	9.8	4.7	125.5	5.4	88.5	7.8	3.7	92	32
6	5	2.3	1	1.68	54	3.6	0.8	58.4	3.5	92.4	6.2	1.4	94	25
7	6	2.8	1-4	5.55		18.3	6.6							
			5-8	5.75		15.6	4.1							
Av.									3.9	91.0	6.0	3.0	94	27

kilogram endogenous uric acid excretion of men. The output of purine bases sometimes appears to approach that of uric acid, but in the more exact determinations (*Experiment 7*) the latter was found to exceed the former three or four times. As to the total daily quantity of purine nitrogen catabolized, its apparent variations are not very considerable. Such as they are, they are no doubt largely the result of irregularities in micturition, and the average of 27 mgm. per kilogram per day is probably a fair enough estimate of the intensity of the guinea-pig's purine metabolism.

The results with the guinea-pig may be compared with those reported by others for another common laboratory rodent, the rabbit. From the figures of Wiechowski¹⁹ one may calculate for this animal uricolytic indices of 83 (Rabbit III) and 94 (Rabbit IV), and a purine coefficient ranging between 20 (Rabbit III) and 38 (Rabbit II). The data of Schittenhelm and Seisser²⁰ yield similar values; the uricolytic index is almost uniformly 95 or 96, and the purine coefficient lies between 20 and 27; bases are equal to or rather less than uric acid. Between rabbit and guinea-pig there is no obvious difference. Whether in both the capacity for

¹⁹ Wiechowski: *loc. cit.*, 1903.

²⁰ Schittenhelm and Seisser: *Zeitschr. f. exp. Path., u. Ther.*, vii, p. 116, 1909.

destroying uric acid always reaches the standard set by the dog, is a point in regard to which the available figures leave one in some doubt. It will require the collection of considerably more analytical material to fully decide the question.

2. The Rat (*Mus norvegicus*)—Family *Muridae*.

The rats made use of were common gray rats trapped locally. In order to obtain sufficient urine for the analyses a large number, of all ages and sizes, were confined in a single cage, and urine was collected for two or more days. Under the circumstances the collection was necessarily far from quantitative, and most of the figures obtained must be taken as minima. The diet consisted of cracker meal, made into pellets, and water. In the determination of purine bases the Krüger-Schmid procedure was adhered to, since none more convenient is available; but the final copper precipitate was here so minute that the determination of its nitrogen cannot lay claim to very great accuracy. For the uric acid this difficulty was eliminated by employing the colorimetric method of Folin and Denis, applied directly to the urine. The allantoin estimations were made by Wiechowski's method in its earliest form, which proved perfectly adapted to the case of rat's urine. In each of the analyses reported the allantoin was actually isolated before its nitrogen was estimated. It separated in large, white, perfectly formed crystals, of the identity or purity of which there could be no doubt. Of these crystals 0.0293 gram, washed but not recrystallized, yielded 10.34 milligrams of nitrogen (Kjeldahl), *i.e.*, 35.3 per cent (calculated for allantoin, 35.4).

All our observations on the rat are included in Table V, in which are recorded the number and the weight of the animals employed in the three experiments that were made.

Discussion. In a recent contribution on the protein metabolism of the rat Folin and Morris²¹ report as "the most striking and interesting feature of the analyses . . . the fact that the urine of rats contains quite as much uric acid in proportion to body weight as does human urine." Their determinations yield averages of from 2.4 to 3.7 mgm. of uric acid nitrogen per kilogram daily.

²¹ Folin and Morris: this *Journal*, xiv, p. 509, 1913.

TABLE V.

The Rat.

EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEIGHT OF ANIMALS kgms.	DAYS OF EXPERIMENT	TOTAL NITROGEN grams	PURINE-ALLANTOIN N					PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin mgms.	Uric acid mgms.	Bases mgms.	Sum mgms.	Per cent of total N	Allantoin	Uric acid	Bases		
8	11	0.86	1-4	2.32	132	3.4	2.9	138.3	6.0	95.4	2.5	2.1	97	40
9	16	2.0	1-2	3.77	192	8.5	6.8	207.3	5.5	92.6	4.1	3.3	96	52
10	21	2.5	1-2	1.75	88	4.2	2.5	94.7	5.3	93.0	4.4	2.6	96	19
Av.									5.6	93.7	3.7	2.7	96	37

Finding the blood also of the rat to be unusually rich in uric acid, they concluded that "the purine metabolism of rats is like that of men and unlike that of other mammals hitherto investigated." This, if true, would be indeed remarkable; but it is a conclusion with which we are unable to agree. Of the accuracy of Folin and Morris's observations we entertain no doubt, and are indeed ready to admit that, if our own rats appeared to excrete uric acid rather less abundantly than theirs—calculation gives a daily output of 1.0 to 2.1 mgm. of nitrogen per kilogram—it may be wholly because the conditions of our experiments were hardly favorable to the quantitative collection of urine. But the uric acid output might have been twice or thrice as great as we found it, it would still have been insignificant compared with the simultaneous output of allantoin. When the latter is determined, the rat is seen at once to constitute no exceptional case. Its urine contains the same products of purine metabolism as that of other rodents, and contains them in the same proportions. Its high output of uric acid is accordingly merely an expression of the fact that the whole purine metabolism, like the protein metabolism or the energy exchange, of so small an animal is pitched at a far higher level than that of man. The guinea-pig too, as we have seen, excretes weight for weight almost as much uric acid as man: but neither in guinea-pig nor in rat does the distribution of purine catabolites in the urine show the slightest approximation to the human type. While man is incapable of destroying uric acid, the uricolytic index of the rat is at least as high as that of related species.

The purine coefficient of the rat cannot be accurately computed from the figures at present available. The discrepancy between the values found is so great that we are forced to attribute it to fortuitous irregularities in the excretion or recovery of the urine. Since the chances of error were mainly in the direction of an underestimate, we think it probable that the highest figures are the ones most likely to be correct. In this opinion we are strengthened by the fact that in the experiments yielding them (8 and 9) the total nitrogen excretion was such as would correspond with the careful estimates of Folin and Morris, while in *Experiment 10* it was much lower. However, until we have performed experiments permitting an exact determination of the rat's daily output, it is probably safest to offer the average (37) as a first approximation to the truth. Even on the basis of this average the rat, which is the smallest animal we have hitherto investigated, exhibits a greater intensity of endogenous purine metabolism than we have encountered in any other species. In this connection it should perhaps be pointed out that many of our rats were evidently very young; the average weight of those in *Experiment 8* was only 78 grams; in *Experiment 9*, 125 grams; in *Experiment 10*, 119 grams. The age of the individual may have an as yet undetermined influence upon the purine coefficient.

III. ORDER MARSUPIALIA.

The Opossum (*Didelphys virginiana*)

Considerable difficulty was experienced in getting with this animal results upon which a reasonable amount of reliance could be placed. All the specimens (six in number), which we had under observation, suffered in health when confined in the laboratory cages. They were exceedingly liable to diarrhoea, and of the numerous diets with which we experimented none was entirely successful in preventing the onset of this annoying symptom. Quite a number of experiments were rendered nugatory on this account. Even when the urine obtained was clean, its analysis presented frequent difficulties. Special purification processes, possibly attended with loss, had to be applied to the allantoin, while the uric acid results, when compared with those of preceding or succeeding periods, were often unaccountably low. Of the large number of

analyses performed we have accordingly selected for report only those in the correctness of which we feel a fair degree of confidence. They are drawn from the results, shown in Table VI, of the following eight experiments.

TABLE VI.
The Opossum.

EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEIGHT OF ANIMALS	DAYS OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PERCENTAGE OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric acid	Bases	Sum	Per cent of total N	Allantoin	Uric acid	Bases		
		kgms.		grams	mgms.	mgms.	mgms.	mgms.						
11	2	6.9	1-2	2.79	52	15.3	4.3	71.6	2.6	72.7	21.4	5.9	77	5.3
		6.7	3-4	2.44	49	—	—	—	—	—	—	—	—	—
		6.6	5-6	1.85	42	—	—	—	—	—	—	—	—	—
12	2	7.1	1-2	3.85	62	23.0	4.8	89.8	2.3	69.1	25.6	5.3	73	6.3
		7.2	1-2	5.17	49	13.0	4.3	66.3	1.3	73.9	19.6	6.5	79	4.6
		7.2	3-4	6.39	33	11.0	—	—	—	—	—	—	75	—
13	2	7.2	5-6	6.31	34	10.0	—	—	—	—	—	—	77	—
		7.5	1	1.73	35	11.0	2	48	2.8	72.9	22.9	4.2	76	6.4
		7.4	2	1.36	30	6.5	3	39.5	2.9	75.9	16.5	7.6	82	5.3
14	2	7.2	3	1.60	22	4.7	2	28.7	1.8	76.6	16.4	7.0	82	4.0
		7.3	1-3	—	—	23.0	5.2	—	—	—	—	—	—	—
		13	1	3.68	41	—	9.2*	50.2	1.4	81.6	—	—	—	3.9
16	4	13	2	2.25	27	—	8.9*	35.9	1.6	75.2	—	—	—	2.8
		13	3	2.68	35	—	9.6*	44.6	1.7	78.3	—	—	—	3.4
		13	1	2.45	34	—	10.4*	44.4	1.8	76.5	—	—	—	3.4
17	4	12.7	2	3.16	29	—	6.9*	35.9	1.1	80.8	—	—	—	2.8
		12.4	3	3.13	25	6.3	2.7	34.0	1.1	73.5	18.5	8.0	80	2.7
		8.5	1-2	2.45	43	5.2	2.8	51	2.1	84.3	10.2	5.5	89	3.0
Av.									1.9	76	19	6	79	4.1

* Includes both uric acid and purine bases.

Experiment 11: Two opossums, a male weighing 4.1 and a female of 2.8 kgm., upon a diet of sweet potatoes and apples. Urine collected every forty-eight hours. The combined weight of the animals fell in six days from 6.9 to 6.6 kgms.

Experiment 12: The same animals at a later period upon apples only.

Experiment 13: The same on a diet of eggs and bread.

Experiment 14: The same animals starved, and urine collected daily.

Experiment 15: The same at a later date, starved for three days, and the whole urine of this period utilized for the more precise determination of the uric acid and purine base output.

Experiment 16: Four other opossums on a bread and milk diet, with daily collection of urine. These animals weighed 2, 2.5, 4 and 4.5 kgms. respectively.

Experiment 17: The same starved for three days.

Experiment 18: Two of the above, weights 4 and 4.5 kgms. on bread and milk; urine of forty-eight hours.

Discussion. The data yielded by the two subjects of *Experiments 11-15* are fairly concordant in showing an allantoin ratio of about 70 to 75, while as much as one sixth to one fourth of the total purine nitrogen catabolized appears as uric acid. The uricolytic index is accordingly decidedly small, ranging for these two animals between 73 and 82, with an average of 78. It would appear, however, that there may be considerable individual variation, for in *Experiment 18* on two other opossums, the uric acid output was relatively smaller and the uricolytic index correspondingly high (89). The divergent result makes it all the more unfortunate that in *Experiments 16* and *17* uric acid determinations, except for one day, are lacking, and that in *Experiment 19* the amount of urine was too small for a satisfactory estimation of allantoin. It is possible that further experience with the opossum would show the ratios of *Experiment 18* to be more fairly representative than the others. Even then we should have to postulate for that animal a feebleness of uricolytic activity than is found in any carnivore or rodent; while if we may rely upon the averages reported in the table, it possesses a lower uricolytic index than any mammal so far examined, excepting man and possibly the horse.²² It would be interesting to ascertain whether other marsupials exhibit the same peculiarity. If they do, their divergent position in evolution would be associated in an interesting way with a departure from the predominant type of purine metabolism.

The precise relation between uric acid and purine bases in the opossums' urine is probably most correctly represented by the data of *Experiment 15*. In this case the bases correspond to less than a fourth of the uric acid output. They were never observed to constitute more than half of the total purines.

Since the urine was collected without catheterization, and the

²² Wiechowski (*Biochem. Zeitschr.*, xix, p. 368, 1909) finds that the horse oxidizes only 50 to 79 per cent of intermediary uric acid; but in our experience horses present a uricolytic index which approaches 90.

opossum micturates at very irregular intervals, it is not possible to draw any exact conclusion as to the daily output of purine-allantoin nitrogen. Its apparent variations are probably accidental only. The figures calculated for the purine coefficient are at least of the same general order of magnitude, and their average (4.1) doubtless represents fairly enough the extent of purine metabolism in this animal. We have seen reason to suspect that the purine coefficient of species, or even of individuals within a species, may exhibit a certain inverse relation to their size. The present instance shows that whatever truth there may be in that notion, size is certainly not the only determining factor. The opossum has a coefficient about eight times smaller than that of dogs of comparable weight. The same thing has been found to be true of the monkey.²³ In both these animals also purine derivatives form but a small proportion of the total nitrogen output. Evidently, leaving size out of the question, there are species, or families, or perhaps whole orders, in which purine metabolism is characteristically more sluggish than in others. The significance of such differences at present escapes us.

²³ Hunter and Givens: *loc. cit.*, 1912. The purine coefficient of our monkey was in the neighborhood of 4.5.

STUDIES IN THE COMPARATIVE BIOCHEMISTRY OF PURINE METABOLISM.

II. THE EXCRETION OF PURINE CATABOLITES IN THE URINE OF UNGULATES.

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In the execution of the program outlined in the preceding paper the ungulates appeared to merit particular attention. Not only does this order include a large number of our domesticated animals, but certain isolated observations, existing already in the literature, indicate that some at least of its members exhibit in one way or another divergences from the common type of purine metabolism. Thus, according to Wiechowski,¹ the horse excretes more uric acid in proportion to its allantoin output, than any other mammal save man (and the chimpanzee); while the urine of the pig² and the horse³ has been found to be richer in purine bases than in uric acid. We have therefore taken opportunity to include in our survey the sheep, the goat, the cow and the horse, in none of which has the simultaneous output of allantoin, uric acid and purine bases hitherto been determined.⁴

¹ Wiechowski: *Biochem. Zeitschr.*, xix, p. 368, 1909.

² Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xlviii, p. 140, 1906; Schittenhelm: *ibid.*, lxvi, p. 53, 1910; Mendel and Lyman: *this Journal*, viii, p. 115, 1910.

³ Schittenhelm and Bendix: *loc. cit.*

⁴ Hart, McCollum, Steenbock and Humphrey (Research Bulletin No. 17, Univ. of Wisconsin Agric. Exper. Station) have published, it is true, figures which show the proportions of these, as of other nitrogenous, substances in the urine of the cow. But their allantoin determinations were made by the method of Poduschka, which is now generally admitted to be unreliable.

1. *The Sheep.*

Our observations upon the purine metabolism of normal sheep are concerned with five different animals, and were made under the following conditions.

Experiment 19. Sheep I, female, starved for four days, with daily collection of urine. The urine was always acid. At the end of the fast the animal weighed 42 kgm.

Experiment 20. Sheep II, female, starved for five days, the urine being collected daily from the second day of fasting. The reaction of the urine was alkaline for the first two days, and afterwards acid. The weight sank from 68.2 kgm. before the fast to 64.5 at the end.

Experiment 21. Sheep III, female, starved for six days, urine being collected daily for the last five. The first sample was neutral, the others acid. The weight at the beginning of the fast was 61, at the close 51.9 kgm.

Experiment 22. Sheep IV, female, weight 39 kgm., upon a diet of corn and green clover. The (alkaline) excretion of one day only was analyzed; it formed approximately a twenty-four-hour specimen.

Experiment 23. Sheep V, female, weighing 28.7 kgm., on oats and alfalfa hay. The single sample of urine analyzed was collected during one day, but represented the output of an unascertained interval, probably greater than twenty-four hours.

The results are shown in Table VII.⁵

Discussion. Nearly all our data for the sheep, it will be seen, apply to the state of inanition. So far as that state is concerned, the results are entirely consistent with one another,⁶ and exhibit the purine metabolism of the sheep as very different from that of carnivores or rodents. The allantoin ratio assumes here smaller values than we have encountered anywhere else among the lower mammals; under certain conditions allantoin may constitute

⁵ The data of the three starvation experiments have already appeared in a report by one of us (Hunter) upon the general nitrogenous metabolism of fasting sheep; see *Quart. Journ. of Physiol.*, vii, No. 1, 1914.

⁶ Results in all respects practically identical have been obtained in three thyroidectomized sheep, also fasting; see Hunter: *Quart. Journ. of Physiol.*, vii, No. 1, 1914. We have not thought it worth while to increase the size of Table VII by their inclusion.

TABLE VII.

The Sheep.

EXPERIMENT NUMBER	SHEEP NUMBER	WEIGHT	DAY OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PER CENT OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric Acid	Bases	Sum	Per cent of total N	Allantoin	Uric Acid	Bases		
		kgms.		gms.	mgms.	mgms.	mgms.	mgms.						
19	I	43.5*	1	8.04	145	28	32	205	2.6	71	14	16	84	4.1
			2	7.64	133	31	29	193	2.5	69	16	15	81	
			3	6.40	108	30	30	168	2.6	64	18	18	78	
			4	5.78	98	24	31	153	2.6	64	16	20	80	
20	II	66.3*	1	10.55	150	27	70	247	2.3	61	11	28	85	3.1
			2	8.46	111	21	55	187	2.2	59	11	29	84	
			3	10.38	115	38	70	223	2.1	52	17	31	75	
			4	6.80	81	22	51	154	2.3	53	14	33	79	
21	III	56.5*	1	8.12	186	59	46	291	3.6	64	20	16	76	4.2
			2	9.02	196	59	55	310	3.4	63	19	18	77	
			3	7.56	165	43	47	255	3.4	65	17	18	79	
			4	4.83	105	30	33	168	3.5	63	18	20	78	
22	IV	39.0	5	5.05	96	30	38	164	3.2	59	18	23	76	6.9
			1	6.42	181	49	38	268	4.2	68	18	14	79	
23	V	28.7	1	6.90	475	60	61	596	8.6	80	10	10	89	21
Average of starvation results.....									2.8	62	16	22	79	3.8
Average of other results..									6.4	74	14	12	84	14
Average of all results.....									3.3	64	16	20	80	8

* Average for experimental period.

little more than half of the sum of purine derivatives in the urine. This peculiarity is partly accounted for by the large output of bases. The precise proportion of the latter is evidently subject to rather wide individual variations; but they are at least rarely inferior in amount to the uric acid, and they may with some sheep be more than twice as abundant. In this respect the sheep presents an analogy with the pig. The smallness of the allantoin ratio is however not altogether due to the magnitude of the base fraction, for uric acid also appears in quantities by no means insignificant. The uricolytic index is accordingly decidedly low; its average value for all starvation urines is 79, so that the sheep, during inanition at least, would appear to oxidize only about

four-fifths of its intermediary uric acid. The daily variations in this respect, apparent with each sheep, are of no very great range. The differences between the three individuals are also not very considerable, being much smaller, for instance, than the differences affecting the output of purine bases. The fasting animals agree further in showing a comparatively low purine coefficient, with an average value of 3.8. This figure is the more reliable in that each experiment was long enough to nearly neutralize the natural irregularity of micturition. It may be noted, as of possible significance, that the lowest coefficient is shown by the biggest sheep.

The results obtained with a sheep upon a diet of corn and clover (*Experiment 22*) are in essential agreement with those upon starving animals. The only difference at all is to be found in a purine coefficient which is indeed higher, yet not by any means disproportionately so. But *Experiment 23*, when the subject was fed upon oats and alfalfa, yields a very different picture. In the first place the purine coefficient is of an altogether different order of magnitude. It is true that we are dealing here with a urine sample that may have represented considerably more than a day's output; but after every allowance on this score has been made, it is clear that in this sheep the excretion of purine derivatives was taking place on a larger scale than in the others.⁷ Further, the excessive output appears almost entirely in the allantoin fraction, and as a consequence the ratios in this sheep have a distinctly altered character. The allantoin ratio is 80, and the uricolytic index almost touches 90. The significance attaching to these divergent values is at present a matter of mere conjecture. They may be nothing more than an expression of individuality, and possibly we should be prepared to encounter variations of this degree among different representatives of a species. But we are not without suspicion that food has been here a factor of some importance. Two possibilities occur to us in this connection. Assuming the values found in the early days of starvation to be representative of the normal endogenous metabolism, those of *Experiment 23* are just such as might have been anticipated, if

⁷ This is evident enough, too, in the relation of purine-derived to total nitrogen.

the diet itself had contained allantoin. We have no evidence at all that oats or alfalfa do contain allantoin, but that substance is certainly a by no means uncommon constituent of vegetable materials.⁸ On the other hand it is possible that the figures of the fast are in reality quite abnormal. There is indeed evidence that starvation tends to a diminution both of the purine coefficient and of the uricolytic index.⁹ It is therefore conceivable enough that the higher ratios of sheep V are those actually representative of a normal state of nutrition. Whatever the relative importance of these various considerations in the case before us, it is clear that in any future series of investigations along the present line, it will be necessary to place all animals upon the same nutritional basis and to actually control the allantoin (as well as purine) content of the food.

It may be pointed out that even a uricolytic index of 89 indicates a capacity for the destruction of uric acid very considerably inferior to that of the dog. A dog excreting, like sheep V, 475 mgm. of allantoin nitrogen would be unlikely to have an output of uric acid nitrogen greater than 25 mgm.

2. *The Goat.*

Two young, but mature, female Angora goats were fed upon oats and alfalfa, and the urine of each was collected at the same hour on two successive days. Except on one occasion, noted in the table, the samples obtained were close approximations to twenty-four-hour lots. In addition we analyzed a mixed specimen of urine obtained at random. Both goats were pregnant. Parturition took place about a month later, when goat I gave birth to one kid, and goat II to two. The data obtained with these goats are to be found in Table VIII.

Discussion. In all five specimens of goat urine the distribution of nitrogen in the three fractions that interest us was practically identical. The output of purine bases is greater than that of uric acid, in which respect the goat resembles the pig and the sheep. In other aspects there is less apparent similarity between

⁸ For the literature on allantoin in plants see Stieger: *Zeitschr. f. physiol. Chem.*, lxxxvi, p. 245, 1913.

⁹ See Hunter: *loc cit.*

TABLE VIII.

The Goat.

EXPERIMENT NUMBER	GOAT NUMBER	WEIGHT	DAY OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PER CENT OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric Acid	Bases	Sum	Per cent of total N	Allantoin	Uric Acid	Bases		
		kgms.		gms.	mgms.	mgms.	mgms.	mgms.						
24	I	50.1	1	20.91	974	88	141	1203	5.8	81	7	12	92	(24)*
			2	19.56	834	86	123	1043	5.3	80	8	12	91	21
25	II	51.0	1	13.27	487	37	83	607	4.6	80	6	14	93	12
			2	14.79	710	50	98	858	5.8	83	6	11	93	17
26†				19.41	951	76	147	1174	6.0	81	6	13	93	
Average.....									5.5	81	7	12	92	17

* Known to represent considerably more than twenty-four hours, therefore not included in average.

† Mixed urine of both goats.

goat and sheep than might have been expected. Our goats show an allantoin excretion far greater, and a uricolytic index much higher, than those of starving sheep of equal size. It is to be noted, however, that when we compare the goats with the one sheep living under the same conditions of diet (*Experiment 23*), the differences all but disappear. It is possible therefore that these differences indicate no essential dissimilarity, but are purely the result of circumstances. One is bound to weigh once more the possibility that the actual diet contained allantoin, or allantoin-yielding materials, or that food in general, apart altogether from its composition, affects the extent and character of purine metabolism. Pregnancy is a circumstance of perhaps still greater significance; it can hardly have been without effect upon the total metabolism of purines, and it may very well have exerted a special influence upon the output of allantoin. Until the actual importance of these possible factors shall have been determined, it is useless to attempt a final comparison between sheep and goat, or to assign to the strictly endogenous purine metabolism of the latter any definite characteristics. Should we for the moment neglect these considerations, and take at their face value the aver-

age results for the goat, we should have to assume for that animal a uricolytic capacity considerable enough, greater perhaps than that of the sheep, yet unmistakably inferior to that of the dog.

3. *The Cow.*

As we did not have at our disposal any of the regular arrangements for metabolism work upon big animals, the urine of the cow had to be obtained by the primitive plan of standing by with a bucket. In spite of this difficulty one of us (O.) succeeded, by dint of the unremitting supervision of a number of animals in their stalls, in collecting two specimens of urine, each of which represented to within thirty minutes the entire excretion of twenty-four hours.¹⁰ The cows were fed meantime upon a measured ration, chiefly of hay and silage. Cow I was well along in the period of lactation, while cow II had given birth to a calf just a week before. They were producing, respectively, about 15 and 27 pounds of milk daily. The data yielded by these animals will be found in the first part of Table IX. The allantoin determinations were made by the most recent form of Wiechowski's method,¹¹ and were performed in duplicate by each of two different observers.

Discussion. The fact that allantoin is a normal constituent of cow's urine was demonstrated ten years ago by Salkowski,¹² but the figures now offered are the first which permit an exact estimate of the quantity actually eliminated. The absolute daily output is seen to be very large, amounting to 26.8 grams in one case and 25.7 in the other, and accounting for 8 and 9 per cent of the total urinary nitrogen. The relation between allantoin and uric acid is such that the uricolytic index of the cow assumes practically the same value (93) as that of the goat. The purine coefficients of these two animals are also of comparable, in fact nearly equal magnitude. The diet was once again

¹⁰ For the opportunity of making these collections we are indebted to the courtesy of Professor H. H. Wing of the Department of Animal Husbandry in this University

¹¹ Wiechowski: Ellinger's *Analyse des Harns*, ii, p. 1076. Wiesbaden, 1913.

¹² Salkowski: *Zeitschr. f. physiol. Chem.*, xlii, p. 213, 1904.

TABLE IX.

The Cow and the Horse.

EXPERIMENT NUMBER	ANIMAL NUMBER	WEIGHT	DAY OF EXPERIMENT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PER CENT OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
					Allantoin	Uric Acid	Bases	Sum	Per cent of total N	Allantoin	Uric Acid	Bases		

Cow.

		kgms.		gms.	gms.	mgms.	mgms.	gms.						
27	I	431	1	90.4	7.56	546	38	8.144	9.0	92.8	6.7	0.5	93	19
28	II	462	1	80.7	7.26	622	62	7.944	9.8	91.4	7.8	0.8	92	17
Average.....									9.4	92.1	7.3	0.7	93	18

Horse.

29	I	597	1	21.9*	0.73	101	3	0.834	3.8	88	120.4	88	7*
			2	58.5	1.41	182	14	1.606	2.7	88	110.9	89	2.7
30	II	608	1	96.8	2.52	349	7	2.876	3.0	88	120.2	88	4.7
Average.....									3.2	88	120.5	88	3.7

* Only a portion of day's urine collected.

of a character that leaves one in doubt whether the figures may be taken as true endogenous values. It is possible, too, that lactation, as well as pregnancy, has important effects upon the metabolism of purines. For the cow therefore, as for the goat, the ratios reported must in the meantime be regarded as provisional only.

From both the goat and the sheep the cow is distinguished by its relatively small output of purine bases. These account for at most a tenth as much nitrogen as the uric acid. Our results confirm in this respect the earlier observations of Schittenhelm and Bendix,¹³ and the more extensive ones of Hart and his co-workers.¹⁴

¹³ Schittenhelm and Bendix: *loc. cit.*

¹⁴ Hart, McCollum, Steenbock and Humphrey: *loc. cit.*

4. *The Horse.*

Two exact twenty-four-hour specimens of horse's urine, together with a third representing only part of a day's output, were obtained by following the plan successful with the cow. The two animals yielding them were healthy geldings in active service at the local fire-station. They received a fixed ration of oats and hay. The allantoin determinations in this case were made in triplicate by three different observers, the latest form of Wiechowski's method being employed. Purine determinations in duplicate were carried out both before and after hydrolysis of the urine. Results in all cases were consistent with one another. The analytical data are reported with those for the cow in Table IX.

Discussion. Wiechowski¹⁵ determined in the urine of two horses the relation between allantoin and uric acid, and calculated the amount of uric acid decomposed as 50 and 79 per cent respectively. With our own horses the proportion of allantoin was decidedly greater, the uricolytic index of each being 88. It must be left to the future to decide whether the divergence between these results expresses a real variability in the species, or whether it is due to other factors not yet elucidated. In any case the horse is seen to possess a rather low order of uricolytic activity, comparable at the best with that of the sheep.

In regard to the relation between uric acid and bases there is between our results and earlier ones an even greater lack of agreement. The proportion of purine bases in our horses' urine was smaller than in any other species which we have examined, so small as to be entirely negligible, and indeed scarcely determinable with exactness. Schittenhelm and Bendix¹⁶ on the other hand found horses' urine extraordinarily rich in bases, so that these stood to uric acid in the high proportion of 8 to 1. A reconciliation of such extremely discrepant reports is quite impossible, and there seems at present to be no escape from the conclusion that the output of purine bases in the horse is subject to enormous individual variations. We have already had occasion, in dealing with the sheep, to note that the base fraction tends to vary within wider limits than the others.

¹⁵ Wiechowski: *loc. cit.*, 1909.

¹⁶ Schittenhelm and Bendix: *loc. cit.*

The purine coefficient of the horse is decidedly low. That of the first horse is in fact lower than has been observed in any other animal save man.¹⁷ This might be taken to confirm our suggestion that, other things being equal, there is a kind of general inverse relation between the intensity of an animal's purine metabolism and its size; and if the gelding exhibits the action of this principle so much better than did the cow, it is perhaps because of its freedom from the influence of any sexual factor such as lactation. But we are admittedly entering here the realm of speculation, and the problems raised await systematic investigation.

5. *The Pig.*

Since Schittenhelm¹⁸ has already provided extensive data upon the purine-allantoin excretion of the pig, we have not thought it necessary to make any observations of our own upon this species.

TABLE X.
The Pig (after Schittenhelm).

PIG NUMBER	WEIGHT	TOTAL NITROGEN	PURINE-ALLANTOIN N					PER CENT OF SAME AS			URICOLYTIC INDEX	PURINE COEFFICIENT
			Allantoin	Uric acid	Bases	Sum	Per cent of total N	Allantoin	Uric acid	Bases		
I	kgms.	gms.	mgms.	mgms.	mgms.	mgms.						
	9.5	5.92	371	6.6	10	387.6	6.5	95.7	1.7	2.6	98	41
II {	7	5.84	236	5	16	257	4.4	91.9	1.9	6.2	98	37
	25.4	5.2	278	6	27	311	6.0	89.4	1.9	8.7	98	12
Average.....							5.6	92.3	1.8	5.8	98	30

In Table X we have quoted the average results for the first five days of each of Schittenhelm's three experiments, and have made therefrom the usual calculations. It should be stated that pig I was six weeks old, pig II eight weeks in the earlier, and four months in the later, experiment. The diet was milk alone in the first two experiments, milk and wheat flour in the last.

¹⁷ One of the horses studied by Wiechowski appears, judging from allantoin and uric acid output alone, to have had a purine coefficient lower still, namely 1.3.

¹⁸ Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxvi, p. 53, 1910.

Discussion. The pig differs from all other ungulates in the size of its uricolytic index, and appears to dispose of uric acid with the same completeness as the dog or the badger. It differs from these animals, and resembles the sheep and goat, in the preponderance of bases over uric acid. The purine coefficient of the young pig is very high, but it becomes smaller as the animal grows. The factor of size has here an easily recognizable influence. In the adult pig the purine coefficient is probably well below 10. Altogether the pig presents a type of purine metabolism not precisely imitated in any other species with which we are acquainted.

General Remarks on the Results with Ungulates.

On a review of the data furnished, in this paper and elsewhere, upon the purine metabolism of the sheep, goat, cow, horse and pig, it becomes evident that we have not succeeded in establishing any general ungulate type. If an excess of bases over uric acid is particularly frequent in this order, it is by no means universal. If the uricolytic index is generally rather low, it is in the pig as high as among carnivora. The purine coefficient is large in some ungulates, small in others, and that without demonstrated reference to any particular circumstances. Nor do the differences noted coincide with any arrangement of ungulate *families*; for within the limits of the Bovidae we meet dissimilarities as striking as those exhibited by any two species whatever. Indeed, if we consider the diverse reports upon the horse, it would seem that the species itself does not always conform to any permanent type. There is no other order which, within the limits of our survey, has shown so much diversity. One is inclined to speculate whether among this group of animals mere domestication and artificial selection have not played an important part in the establishment of variations from the commonest mammalian type.

GENERAL RÉSUMÉ.

A general summary of the results reported in this and the preceding paper is to be found in Table XI. This table includes not only the averages for the twelve species which have been specially investigated in this laboratory, but also repre-

sentative data from other sources upon five other mammals. Among the seventeen species thus brought under consideration there are three—all of the order of Primates—that have not yet been mentioned. These are the monkey, the chimpanzee, and man. The exceptional position of the last in regard to purine metabolism is well enough known, and formed indeed the starting point of the present investigation; with man the power of oxidizing uric acid has been completely, or all but completely lost, and even the insignificant quantity of allantoin that his urine generally contains¹⁹ is perhaps of exogenous origin only. The chimpanzee has lately been found to conform in this respect to the human type,²⁰ an observation which from our present point of view is of very great interest indeed. The case of the monkey has been discussed at length in recent communications from this laboratory,²¹ in which it has been shown that that animal excretes bases more abundantly than uric acid, and has a uricolytic index in the neighborhood of 90. The monkey can therefore hardly be said to show any approximation to the extreme type of purine metabolism exemplified by man and chimpanzee; its closest affiliations are in fact, curiously enough, with the ungulates, and especially with the sheep.

Many of the ratios exhibited in Table XI have as yet no claim to be regarded as definite standards. We have indeed taken frequent occasion to point out that the investigations upon which they are based are in many respects but preliminary surveys; and possibly the most important result of our work is to indicate the route that must be followed in establishing the standards that we sought. It will be necessary in the future to carefully discriminate the influence exerted upon endogenous purine metabolism by a number of factors—size, food, sexual activities, and so on—the importance, or lack of importance, of which we are not yet able to estimate. It will be necessary also to greatly increase the range of our statistical material, and to determine the limits within which individuality may make itself felt. Yet

¹⁹ Wiechowski: *loc. cit.*, 1909, and *Biochem. Zeitschr.*, xxv, p. 431, 1910; Schittenhelm and Wiener: *Zeitschr. f. physiol. Chem.*, lxiii, p. 283, 1909; Ascher: *Biochem. Zeitschr.*, xxvi, p. 370, 1910; Fairhall and Hawk: *Journ. Amer. Chem. Soc.*, xxxiv, p. 546, 1912.

²⁰ Wiechowski: *Prager med. Wochenschr.*, 1912, p. 275.

²¹ Hunter and Givens: this *Journal*, xiii, p. 371, 1912; and xvii, p. 37, 1914.

TABLE XI.

ORDER AND SPECIES	PER CENT OF PURINE-ALLANTOIN NITROGEN			URICOLYTIC INDEX	PURINE COEFFICIENT
	Allantoin	Uric acid	Bases		
<i>Marsupialia</i>					
Opossum.....	76.0	19.0	6.0	79	4.1
<i>Rodentia</i>					
Rabbit.....				95	26.0
Guinea-pig.....	91.0	6.0	3.0	94	27.0
Rat.....	93.7	3.7	2.7	96	37.0
<i>Ungulata</i>					
Sheep.....	64.0	16.0	20.0	80	8.0
Goat.....	81.0	7.0	12.0	92	17.0
Cow.....	92.1	7.3	0.7	93	18.0
Horse.....	88.0	12.0	0.5	88	3.7
Pig.....	92.3	1.8	5.8	98	12.0
<i>Carnivora</i>					
Raccoon.....	92.6	5.4	2.0	95	16.0
Badger.....	96.9	1.9	1.2	98	28.0
Dog.....	97.1	1.9	1.3	98	29.0
Coyote.....	95.6	2.6	1.8	97	23.0
Cat.....				97	
<i>Primates</i>					
Monkey.....	66.0	8.0	26.0	89	4.5
Chimpanzee.....				0	
Man.....	2.0	90.0	8.0	2(?)	2.5

even the incomplete and in part provisional data we have already accumulated, offer, we venture to believe, sufficient justification for the following conclusions:

1. There exist among mammalia several distinct types of purine metabolism. 2. Carnivora and rodents exhibit a type in which "uricolysis" is practically complete, though possibly somewhat less so in the latter than in the former. 3. Most ungulates, yet apparently not all, have a capacity for uric acid oxidation inferior to that of the orders just mentioned. 4. The opossum excretes relatively more uric acid in relation to its allantoin output, than any other mammal except the higher primates. 5. The only species in which allantoin is not quantitatively the most important product of purine metabolism are man and the anthropoid apes. 6. While in the majority of species uric acid is excreted in greater abundance than purine bases, there are instances, especially frequent among the un-

gulates, where the reverse is true; the precise relation between the two is, even for the species, not so nearly constant as the uricolytic index. 7. The members of one order sometimes do (carnivora, rodents), and sometimes do not (ungulates, primates), all conform to a single one of the types indicated; occasionally the existence of special family types is suggested. 8. No obvious system of evolutionary development of the various types has as yet revealed itself; it can however be said that widely divergent types are usually located in widely separated orders (compare, for instance the opossum with the rat, or man with the carnivora). 9. The total endogenous purine metabolism per unit of weight is in a general way inversely related to the size of the animal; but there are many striking exceptions, and many other factors evidently play a part in determining this relation.

The comparative biochemistry of purine metabolism may be, and has been, studied by many other methods than that which we have followed. Schittenhelm, Jones, and others have for instance investigated the enzymes effective in the process, and their distribution in the organs of different animals;²² while Folin,²³ and Bass and Wicchowski²⁴ have attacked the problem from the point of view of blood analysis. We do not feel that it would be profitable as yet to discuss in detail the bearing which the results of these investigators have upon our own. It is self-obvious that the results of all methods must ultimately prove susceptible of combination into a harmonious picture. In the case of man they may be said to have done so already; the practical absence of allantoin from his urine is readily coördinated with the absence of uricase from his tissues, and the unusual richness of his blood in uric acid. It is not so easy, on the data available, to see why the horse should excrete a greater proportion of uric acid than the dog, or why the monkey's urine should be richer in purine bases than that of the cow. Yet it cannot be doubted that, as our information along every line of inquiry increases, each special case will receive in turn a rational explanation founded on experimental evidence.

²² The chief results attained by this method will be found in *Oppenheimer's Handbuch der Biochemie*, iv, p. 506, Jena, 1911.

²³ Folin and Denis: this *Journal*, xiv, p. 29, 1913.

²⁴ Bass and Wicchowski: *Wiener klin. Wochenschr.*, xxv, p. 1863.

BRIEF NOTES CONCERNING ALLANTOIN.

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In the course of the past few years many series of allantoin determinations have been made in this laboratory in the investigation of the metabolism of purines.¹ In connection with these the method of Wiechowski² for the determination of allantoin was used. In order to control the same, I have performed a number of experiments dealing with the behavior of allantoin under a variety of conditions. Some of the questions involved are such as will almost inevitably occur to others engaging in extended work of a similar character. The rather disconnected observations that follow are therefore recorded as being perhaps not altogether destitute of practical interest, and as possibly throwing some light on past work. They are taken up in the following order:

(a) The rate of disappearance of allantoin from pure aqueous solutions.

(b) The destruction of allantoin by alkalies.

(c) The disappearance of allantoin from urine.

(d) Some points in connection with the determination of allantoin in urine.

(a) *The rate of disappearance of allantoin from pure aqueous solutions.*

Wiechowski³ states that allantoin is not stable in pure aqueous solution. He found a 0.1 per cent allantoin solution after standing a few weeks ("nach wochenlangem Stehen") in a closed flask,

¹ Hunter and Givens: this *Journal*, xiii, p. 371, 1912; *ibid.*, xvii, p. 37, 1914.

² Wiechowski: *Beitr. z. chem. Physiol. u. Path.*, xi, p. 109, 1907.

³ Wiechowski: *Zeitschr. f. physiol. Chem.*, xxv, p. 453, 1910.

moulds being absent, free from allantoin. Under similar conditions I have noted some disappearance of allantoin, but neither so rapid nor so complete as Wiechowski reports.

The following allantoin solutions varying in concentration were kept in closed flasks and analyzed at various times by taking a known quantity, precipitating with Wiechowski's reagent, and estimating the nitrogen in the precipitate (Kjeldahl).

Allantoin 1:0.6556 gram pure allantoin dissolved in 500 cc. distilled water May 16, 1912.

Allantoin 2:1.1396 gram pure allantoin dissolved in 250 cc. distilled water May 24, 1912.

Allantoin 3:2.421 gram pure allantoin dissolved in 1000 cc. distilled water Jan. 28, 1913.

Allantoin 4:0.260 gram pure allantoin dissolved in tap water Feb. 18, 1914.

They were analyzed in 25 cc.-lots calculated to contain in 1, 11.58 mgm. N; in 2, 40.0 mgm. N; in 3, 21.38 mgm. N; in 4, 11.49 mgm. N.

Analysis of 1.

Date	Mgm. N Found:	Date	Mgm. N Found:
May 16, 1912.....	$\left\{ \begin{array}{l} 11.59 \\ 11.42 \end{array} \right\} 11.50$	Aug. 12, 1913.....	$\left\{ \begin{array}{l} 11.32 \\ 11.27 \end{array} \right\} 11.30$
Oct. 24, 1913.....	$\left\{ \begin{array}{l} 10.96 \\ 10.96 \end{array} \right\} 10.96$	Jan. 27, 1914.....	$\left\{ \begin{array}{l} 10.78 \\ 10.74 \end{array} \right\} 10.76$

Analysis of 2.

Date	Mgm. N Found:	Date	Mgm. N Found:
May 24, 1912.....	$\left\{ \begin{array}{l} 39.8 \\ 39.8 \end{array} \right\} 39.8$	Sept. 28, 1912.....	39.2
Oct. 24, 1912.....	39.2		

Analysis of 3.

Date	Mgm. N Found:	Date	Mgm. N Found:
Jan. 28, 1913.....	$\left\{ \begin{array}{l} 21.24 \\ 21.10 \end{array} \right\} 21.17$	Aug. 10, 1913.....	$\left\{ \begin{array}{l} 20.83 \\ 20.96 \end{array} \right\} 20.89$
Oct. 24, 1913.....	20.55	Jan. 27, 1914.....	$\left\{ \begin{array}{l} 20.27 \\ 20.40 \end{array} \right\} 20.38$

Analysis of 4.

Date	Mgm. N Found:	Date	Mgm. N Found:
Feb. 18, 1914.....	$\left\{ \begin{array}{l} 11.52 \\ 11.46 \end{array} \right\} 11.49$	Mar. 4, 1914.....	$\left\{ \begin{array}{l} 11.37 \\ 11.37 \end{array} \right\} 11.37$
Mar. 23, 1914.....	$\left\{ \begin{array}{l} 10.71 \\ 10.79 \end{array} \right\} 10.75$		

From the above we are able to construct the following table:

SOL'N	STRENGTH	AGE	ACTUAL LOSS IN MG. N	LOSS OF TOTAL
	<i>per cent</i>	<i>days</i>		<i>per cent</i>
1	0.13	90	4	1.7
		525	10.8	4.7
		620	14.8	6.3
2	0.45	120	6	1.5
		415	6	1.5
3	0.24	194	11.2	2.0
		270	24.8	4.4
		365	31.6	5.7
4	0.13	14	1.0	1.08
		24	5.9	6.4

From the foregoing it seems without a doubt that there is a slow spontaneous destruction of allantoin. There is less destruction the more concentrated the solution. This might be taken to mean that some of the allantoin in the weaker solutions was destroyed by bacteria. I do not think this probable for according to Coppin⁴ a solution of allantoin made of distilled water is not favorable to cell division and growth, at least in plants. Wiechowski does not state whether he used tap or distilled or sterile water in his solutions. That our tap water here would be more favorable to destruction of allantoin because it comes nearer to being a medium for bacterial growth and because of its alkalinity, seems indicated from solution 4. How far the destruction has proceeded in the above cases I am not prepared to state, though the following might indicate that it had possibly gone as far as ammonia. 25 cc. of solutions 1 and 2 were distilled with 1 gm. MgO until no more NH₃ was coming off and from 1 was received 0.77 mgm. N and from 2 0.63 mgm. N. This amount of nitrogen almost quantitatively corresponds to the loss on standing of allantoin N in 25 cc. of these solutions, which was in 620 days for 1 0.74 mgm. N and for 2 in 415 days 0.79 mgm. N. This is not due merely to a splitting off of ammonia from allantoin by the magnesia; for when two 25-cc. portions of a *freshly prepared* solution of allantoin

⁴ Coppin: *Biochem. Journ.*, vi, p. 416, 1912.

containing 20.92 mgm. N diluted to 150 cc. were distilled for 20 minutes with 1 gm. MgO no ammonia was given off.

(b) *The destruction of allantoin by alkalies.*

While allantoin appears, therefore, to be relatively stable in neutral solutions, it is rapidly destroyed by alkalies.⁵ This is indeed well known and has recently been specially emphasized by Wiechowski.

To two 25 cc. samples of allantoin containing 21.24 mgm. N were added 23.94 cc. of $\frac{N}{10}$ KOH and the mixtures were allowed to stand for a month at room temperature. Upon analysis they contained 1.9 and 2.1 mgm. N, that is, 90.2 per cent and 91.1 per cent loss. A much more striking loss is that of 43 per cent caused by 20 cc. $\frac{N}{10}$ KOH in twenty-four hours at 38°C. (see section d).

Boiling allantoin with baryta or MgO will liberate very little if any ammonia, but that destruction nevertheless takes place is evident from the following:

MGM. ALLANTOIN N	DISTILLED FOR 30 MINS. WITH	NH ₃ GIVEN OFF AS N	ALLANTOIN RECOVERED FROM RESIDUE IN MGM. N
20.92	{ 5 cc. saturated	0.32	9.9 } 9.75 9.6 } 3.2 4.3
20.92	{ Ba(OH) ₂	0.27	
41.84	{ 10 cc. saturated	0.73	
41.84	{ Ba(OH) ₂	0.71	
22.22	1 gm. MgO	0.11	
22.22		0.11	
22.22	{ 5 cc. saturated	0.77	
22.22	{ Ba(OH) ₂	0.66	

Thus it is evident from the above that allantoin is rapidly destroyed by boiling with baryta or MgO. The loss with the former was 85.6 per cent and 80.7 per cent; with the latter 55.5 per cent and 56.8 per cent.

(c) *Destruction of allantoin by fecal bacteria.*

When allantoin is fed by mouth it is usual to recover in the urine only a fraction of the dose. By some authors this is taken to mean that the allantoin is destroyed in intermediary metab-

⁵ Wiechowski: *Ellinger's Analyse des Harns*, ii, p. 1072, Wiesbaden, 1913.

olism. Against such an explanation it can be urged that the excretion of parenterally introduced allantoin is practically quantitative. Wiechowski⁶ accordingly argues that the destruction of ingested allantoin probably precedes its absorption. As destructive agents probably operative in the intestines he suggests warmth, alkalinity, and bacteria. Hunter and Givens⁷ have accepted this view as explaining the deficit of allantoin after feeding that substance to their monkey. While, however, the effectiveness of an alkali in destroying allantoin is sufficiently proved, there is no direct evidence that the intestinal bacteria are capable of any extensive activity in this direction. I have accordingly tested the matter, making use for the purpose of the fecal bacteria of the above mentioned monkey.

A suspension of feces was made in sterile distilled water and filtered through glass wool. To 50 cc. of this suspension 25 cc. of a fresh solution of allantoin in sterile distilled water containing 0.121 gram allantoin = 42.98 mgm. N were added. The flasks were then incubated for twenty-four hours when they were neutralized with $\frac{N}{10}$ sulphuric acid, requiring 23.67 and 21.48 cc. They were treated with basic lead acetate, the excess of lead removed with H_2S and the latter with air. No further treatment was necessary, as the fluid did not react with phosphotungstic acid nor silver. They were neutralized and precipitated with Wiechowski's reagent and the crystals isolated and weighed as follows: 0.0126 and 0.0117 (average 0.0121) gram allantoin; that is a loss of 90 per cent.

The above loss was not due merely to alkalinity. This is shown by the following control.

To four flasks were added 25 cc. of the above allantoin solution. 1 and 2 had in addition 20 cc. $\frac{N}{10}$ KOH. All four flasks were incubated for twenty-four hours at 38°C. At the end of this time they were neutralized and precipitated with Wiechowski's reagent, and nitrogen estimations were made with the following results:

1. 23.95	} 24.00 mgm. N.	3. 42.38	} 42.39 mgm. N.
2. 24.05		4. 42.40	

That is to say 43.4 per cent of the allantoin was destroyed in twenty-four hours by 20 cc. of $\frac{N}{10}$ KOH at body temperature.

If 90 per cent was destroyed by the combined action of the alkalinity produced and the bacteria, then 46.6 per cent was destroyed by bacteria.

⁶ Wiechowski: *loc. cit.* 1910.

⁷ Hunter and Givens: *loc. cit.*, 1912.

(d) *The disappearance of allantoin from urine.*

Salkowski⁸ was the first to notice the spontaneous destruction of allantoin, with a consequent increase of oxalic acid, in cow's urine. Minkowski⁹ noticed in working with the mother liquor of his allantoin solutions from dog's urine that if he precipitated with calcium and then digested with sodium hydroxide, on the water bath, he received a noticeable increase of oxalic acid. Wiechowski¹⁰ observed the disappearance of allantoin in horse urine (native alkaline) after it had stood a few days in the laboratory over chloroform.

I have noticed the more or less complete destruction of allantoin in some urines. A sample of coyote urine kept for three years contained but a trace of allantoin. At the end of this time the alkalinity of 250 cc. of this urine was equivalent to 1030 cc. of $\frac{N}{10}$ KOH, which was no doubt due mostly to ammonium carbonate. 250 cc. of this urine were worked up by our modification¹¹ of Wiechowski's method for determining allantoin in monkey urine. Only 19 mgm. of typical allantoin crystals were obtained, which melted alongside of pure allantoin at 234°C. (uncorr.). This same amount of urine when fresh contained about 0.45 gram allantoin.

On the other hand in urines which have been acidified and well preserved the destruction of allantoin takes place more slowly. Some cow urine was preserved three months with sulphuric acid and thymol. At the time of collection of the twenty-four-hour sample of 5715 cc. there were found 7.27 grams of allantoin N, and three months later 6.89 grams of allantoin N; that is, 6 per cent at the most disappeared in ninety days.

Again in a twenty-four-hour sample of horse urine of 7250 cc. preserved with acetic acid and thymol there were found 1.34 grams of allantoin N and one month later 1.32 grams of allantoin N. The slight difference I am more inclined to attribute to the limitations of the method than to destruction of allantoin.

⁸ Salkowski: *Zeitschr. f. physiol. Chem.*, xlii, p. 213, 1904.

⁹ Minkowski: *Arch. f. exp. Path. u. Pharm.*, xli, p. 396, 1898.

¹⁰ Wiechowski: *loc. cit.*, 1910.

¹¹ Hunter and Givens: *loc. cit.*, 1912.

(e) *Some points in connection with the determination of allantoin in urine.*

The method of allantoin determination offered by Wiechowski possesses over all earlier ones the decisive advantage of at least approximate accuracy. Unfortunately it is one that calls for a considerable expenditure of time. When a long series of determinations has to be made this becomes a serious disadvantage. I have therefore made many attempts to shorten the method without lowering its accuracy. It is useless to report these here, since the successful modifications to which they led have been independently adopted by Wiechowski himself in the most recently published account of his method.¹²

One point was however ascertained which may occasionally prove to be useful. Once the allantoin has been precipitated by the mercuric acetate-sodium acetate reagent, the precipitate may be allowed to stand almost indefinitely without being affected by the alkalinity of the precipitating solution. Three sets of duplicates of allantoin were precipitated at the same time with this reagent and allowed to stand.

Milligrams of N found at once	40 days later	190 days later
21.24 } 21.17	21.43 } 21.47	21.01 } 21.08
21.10 }	21.51 }	21.15 }

It would seem feasible therefore, in the course of a long series of determinations, to carry each one at first only to the point of the first precipitation of allantoin, and to set the precipitates then aside until it should be convenient to collect and estimate the nitrogen in all at one time.

It would seem also from the data of section (d) that the treatment of the sample of urine need not be begun immediately after its collection. If it is made decidedly acid, kept cool, and efficiently preserved, there will be no appreciable loss of allantoin within a month.

If one uses Wiechowski's new PbO method¹² it does not seem safe to allow the urine to stand after making alkaline with MgO. Some horse urine was cleaned by this method and two samples precipitated at once and one allowed to stand forty-eight hours

¹² Wiechowski: *Ellinger's Analyse des Harns*, ii, p. 1076, Wiesbaden, 1913.

and then precipitated. Analysis at once showed 4.51 and 4.69 mgm. allantoin N; analysis after standing forty-eight hours, 3.08 mgm. allantoin N.

If one uses mercuric nitrate as a precipitant it does not seem necessary to neutralize afterwards as Ackroyd did.¹³ He does not state how much nitric acid he used in making up his mercuric nitrate reagent. The reason that it was unnecessary to neutralize in our case may be due to the fact that we used a minimal amount of nitric acid in making the reagent, about 5 cc. of nitric acid per 100 to make up 20 per cent mercuric nitrate. Three sets of duplicates containing 11.58 mgm. allantoin N were precipitated as follows:

With Wiechowski reagent 1a and 1b; with 8 cc. of 20 per cent mercuric nitrate 2a and 2b; 3a and 3b precipitated as 2a and 2b and then neutralized. In estimating nitrogen in 2 and 3 any HNO₃ not washed out was first driven off before adding copper sulphate and potassium sulphate.

Milligrams of nitrogen found

1a.....11.59	} 11.50	2a.....11.67	} 11.42	3a.....11.03	} 11.45
1b.....11.43		2b.....11.16		3b.....11.86	

¹³ Ackroyd: *Biochem. Journ.*, v, p. 400, 1911.

CONCERNING THE ORGANIC PHOSPHORIC ACID COMPOUND OF WHEAT BRAN. II.

NINTH PAPER ON PHYTIN.

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INTRODUCTION.

It has been shown in a preliminary report¹ from this laboratory that the composition of the organic phosphoric acid isolated from wheat bran is different from that of phytic acid or inosite phosphoric acid which is present in other grains and seeds. Patten and Hart² who first investigated this substance from wheat bran came to the conclusion that it was identical with the "anhydroxymethylene diphosphoric acid" of Posternak. The analysis which they report of the acid preparation which they had isolated was in close agreement with the calculated composition of the above acid.

We suggested in our earlier report³ that the acid analyzed by the above authors must have been contaminated with inorganic phosphoric acid because wheat bran contains appreciable quantities of inorganic phosphate and by their method of isolation both the organic and the inorganic phosphates would be precipitated at the same time.

The substances which we prepared from wheat bran and analyzed⁴ were free from inorganic phosphate, *i.e.*, they gave no precipitate with ammonium molybdate solution. In order to obtain

¹ This *Journal*, xii, p. 447, 1912; Tech. Bull. 22, N. Y. Agric. Exp. Station, 1912.

² *Amer. Chem. Journ.*, xxxi, p. 566, 1904.

³ *Loc. cit.*

⁴ *Loc. cit.*

preparations free from inorganic phosphate we found it necessary to precipitate the substance repeatedly from very dilute hydrochloric acid with about an equal volume of alcohol. By this method inorganic phosphates are removed because they are more soluble in the dilute acid alcohol mixture than are the salts of the organic phosphoric acid.

In this way we obtained a crude preparation of the organic phosphorus compound which was readily soluble in cold water. Combined with it were various bases, calcium, magnesium, potassium, sodium, etc., and also some substance which contained nitrogen. By treating this crude substance in aqueous solution with barium hydroxide the above mentioned bases as well as the nitrogen-containing compound were eliminated. The resulting insoluble barium salts were amorphous and could not be obtained in crystalline form. These salts did not have the composition of barium phytates but agreed approximately with the formulas $C_{25}H_{55}O_{54}P_9Ba_5$ and $C_{20}H_{45}O_{49}P_9Ba_5$; from both salts an acid was isolated which approximately agreed with the formula $C_{20}H_{55}O_{49}P_9$. All the various preparations which were prepared in various ways agreed with the above formulas but since the substances were all amorphous we stated particularly⁵ that, "the empirical formulas suggested in this paper are of course purely tentative."

Although we begged to reserve the further study of this organic phosphoric acid compound as well as the nitrogen containing substance Rather⁶ in a recent paper reports some work on the same subject. This author had isolated some crude acid preparations from cottonseed meal and wheat bran from which silver salts were prepared. It is claimed that these silver precipitates are pure homogeneous compounds and that they are salts of an organic phosphoric acid of the formula $C_{12}H_{41}O_{42}P_9$. Since these results did not agree with those of any previous investigator in this field, the author concludes that his results are the only correct ones; owing to his superior method of isolation and purification, purer products had been obtained and he proposes the

⁵ *Loc. cit.*

⁶ *Journ. Amer. Chem. Soc.*, xxxv, p. 890, 1913; *Bull.* 156, Texas Agric. Experiment Station, 1913.

formula $C_{12}H_{41}O_{42}P_9$ as the correct one for phytic acid or inosite phosphoric acid.

We have already shown⁷ that the above author is in error in respect to the composition of the acid in cottonseed meal. The carefully purified and many times recrystallized barium salts which we prepared from the acid from cottonseed meal had the composition of acid barium salts of inosite hexaphosphate, $C_6H_{12}O_{24}P_6Ba_3$ and $(C_6H_{11}O_{24}P_6)_2Ba_7$. The free acid prepared from these salts corresponds to inosite hexaphosphate, $C_6H_{18}O_{24}P_6$, and not to an acid of the formula $C_{12}H_{41}O_{42}P_9$.

The silver precipitates which we prepared from the above inosite hexaphosphate were pure white amorphous substances and very slightly sensitive to light. We showed,⁸ however, that these silver precipitates are not homogeneous salts of inosite hexaphosphate but mixtures of more or less acid salts of the above acid.

In again taking up the investigation of the organic phosphorus compounds of wheat bran we have first of all critically repeated our former work. The results completely confirm those reported in our earlier paper.⁹ We then repeated the work of Rather, following his method of isolating the crude acid as closely as possible. The acid preparation obtained in this way was divided into two parts: One portion was used for the preparation of the silver salt as described by the above author; the other portion was transformed into the barium salt and purified in accordance with our previous method.

The barium salts which were obtained in this way were found to agree very closely in composition with those previously reported, viz., $C_{20}H_{45}O_{49}P_9Ba_5$ and not with salts of an acid of the formula $C_{12}H_{41}O_{49}P_9$.

The silver precipitates obtained from this crude acid preparation varied in composition according to the method of preparation but in one case the substance had approximately the composition stated by Rather. A simple examination of these silver precipitates quickly revealed the fact, however, that they were not pure homogeneous salts of an organic phosphoric acid of the formula

⁷ This *Journal*, xvii, p. 141, 1914.

⁸ *Ibid.*, p. 149.

⁹ This *Journal*, xii, p. 447, 1912; Tech. Bull. 22, N. Y. Agric. Exp. Station, 1912.

$C_{12}H_{41}O_{42}P_9$ as claimed by the above author but that they were largely contaminated with inorganic silver phosphate—varying from 42 to 90 per cent.

In our first report¹⁰ on this subject we called attention to the fact that wheat bran extracts contain relatively much inorganic phosphate. This part of our paper seems to have escaped the attention of Rather. This author like Patten and Hart has made no provision for eliminating inorganic phosphate in his method of isolating the organic phosphoric acid.

Since inorganic and organic phosphoric acids are both present in the crude acid prepared in accordance with the methods of the above authors naturally the silver precipitates obtained from such acid neutralized with ammonia must contain both inorganic and organic silver phosphates because both are only slightly soluble in neutral aqueous solution.

Although the silver precipitates obtained from cottonseed meal and wheat bran may have approximately the same composition it is surprising that anyone could consider them identical for so far as the most obvious physical property, viz., appearance, is concerned they are entirely dissimilar—the silver precipitates from the inosite hexaphosphate from cottonseed meal are, as already mentioned, of pure white color and they are very slightly affected by light. The silver precipitates obtained from the acid from wheat bran, on the other hand, are only white at the moment of precipitation. These substances are either extremely sensitive to light or else the silver becomes reduced for the color rapidly darkens and finally turns quite black. Even when working under careful exclusion of direct light we have been unable to obtain a white silver preparation from the wheat bran compound.

While the amorphous barium salts, prepared as will be mentioned later, from the organic phosphorus compound of wheat bran, show a close agreement in composition with those reported previously,¹¹ we do not believe that they are homogeneous compounds. We have been able to separate these amorphous precipitates into several fractions some of which were semi-crystalline but the composition was not constant. In no case, however, have we been able

¹⁰ *Loc. cit.*

¹¹ *Loc. cit.*

to obtain a trace of a salt having the composition of inosite hexaphosphate.

From the results which we have obtained it appears probable that these amorphous barium precipitates are mixtures, probably of various organic phosphoric acids. Some of these are undoubtedly lower phosphoric acid esters of inosite but it is possible that phosphoric acid esters of other carbohydrates are present.

Neither Patten and Hart nor Rather mention the presence of oxalic acid in the preparations from wheat bran which they examined. It would seem, however, from our results that the crude substance obtained by precipitating a dilute hydrochloric acid extract of wheat bran with alcohol contains rather large quantities of oxalates.

The removal of this oxalate presented greater difficulties than the elimination of inorganic phosphate. As a barium salt oxalic acid is precipitated at every stage along with the salts of the organic phosphoric acid. It is likewise carried down in the precipitate obtained with copper acetate because copper oxalate is very slightly soluble. The complete removal of barium oxalate from the other mixture of barium salts finally succeeded by allowing it to crystallize out from a very dilute hydrochloric acid solution of the mixed salts.

If the name "phytin" is to be applied to certain salts of inosite hexaphosphate then it is evident that wheat bran does not contain phytin for we have been unable to isolate any salt of this acid from this material. It appears more probable that wheat bran contains several different organic phosphoric acids. So far we have been able to identify only one of these acids, viz., inosite monophosphate, a substance which will be described in a succeeding paper.

EXPERIMENTAL PART.

Isolation of the crude organic phosphorus compound from wheat bran.

A larger quantity of the crude natural organic phosphorus compound was prepared by precipitating a 0.2 per cent hydrochloric acid extract of wheat bran with alcohol exactly as before. From 25 kgm. of wheat bran we obtained 222 grams of the crude

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compound as a nearly white amorphous powder. This substance had the following composition:

C = 20.21; H = 3.54; P = 13.45; Mg = 8.20; K = 5.23; Na = 2.56; Ca = trace.

Nitrogen was present but it was not determined. The substance was practically free from inorganic phosphate as it gave only a trace of a yellow precipitate on warming its nitric acid solution to 65° for a longer time with ammonium molybdate.

Of the above substance, 50 grams were suspended in a little water and dissolved by the addition of a few drops of hydrochloric acid. After diluting with water it was precipitated by adding barium hydroxide in excess. It was then filtered and thoroughly washed in water.

Preparation of the nitrogen-containing substance.

The filtrate from the above was freed from excess of barium hydroxide with carbon dioxide, filtered and evaporated in vacuum at a temperature of 40°–45° to small bulk and again filtered from a small amount of barium carbonate. The concentrated solution was then poured into about 500 cc. of alcohol. It separated as a somewhat sticky mass which soon hardened. It was filtered, washed in alcohol and ether and dried in vacuum over sulphuric acid. It weighed 7 grams. It was dissolved in a small amount of water and reprecipitated with alcohol, filtered, washed and dried as before. It was obtained as a nearly white amorphous powder. The substance was very soluble in water and it was free from chlorides and inorganic phosphate.

It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

Found: C = 39.22; H = 5.43; N = 14.26; Amino nitrogen by the Van Slyke method = 0.999; Organic phosphorus after decomposing by the Neumann method = 0.875; Ba = 10.43 and it also contained small quantities of magnesia, potash and soda.

Preparation of the crude barium salt of the organic phosphorus compound.

The insoluble precipitate obtained with barium hydroxide from the 50 grams of the crude substance was again precipitated three

times with barium hydroxide and four times with alcohol alternately from 0.5 per cent hydrochloric acid. After drying in vacuum the product was a white amorphous powder which weighed 22 grams.

A preliminary experiment showed that when this substance was dissolved in a little 0.5 per cent hydrochloric acid and then mixed with a concentrated solution of barium chloride and allowed to stand that some crystalline precipitate separated. The whole of the above barium salt was therefore dissolved in the least possible amount of 0.5 per cent hydrochloric acid; 5 grams of barium chloride dissolved in a little water was added and the whole allowed to stand for twenty-four hours. A heavy white crystalline powder had then separated. This was filtered and washed several times in water and finally in alcohol and ether and dried in the air. This crystalline substance was later found to consist principally of barium oxalate. It weighed 2.75 grams which is equal to 12.5 per cent of the barium salt used.

The filtrate, after removing the above crystals, was precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. It was again dissolved in the minimum quantity of 0.5 per cent hydrochloric acid, mixed with barium chloride and allowed to stand for twenty-four hours. A small amount of a crystalline precipitate had separated which was filtered, washed, and dried. The filtrate was again precipitated with alcohol, filtered, washed and dried and again dissolved in 0.5 per cent hydrochloric acid, mixed with barium chloride and allowed to stand as before but no precipitate separated. It was then precipitated with alcohol, filtered, washed and dissolved in 0.5 per cent hydrochloric acid and again precipitated with alcohol. It was finally filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white amorphous powder, free from chlorides and inorganic phosphate. On analysis, after drying at 105° in vacuum over phosphorus pentoxide, the following result was obtained:

C = 14.98; H = 2.46; P = 11.89; Ba = 31.64 per cent.

The substance was quite soluble in cold water. It was therefore rubbed up in a mortar with a small quantity of water in which

the greater portion dissolved. After standing over night the insoluble substance was filtered, washed in water, alcohol and ether and dried in vacuum over sulphuric acid. The water-soluble portion was precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum as above.

After drying at 105° as before the following results were obtained on analysis:

The water-insoluble portion gave: C = 12.58; H = 2.02; P = 10.06; Ba = 40.62 per cent.

The water-soluble portion gave: C = 15.54; H = 2.95; P = 12.30; Ba = 30.24 per cent.

Examination of the crystalline barium oxalate obtained from the above barium salt.

The substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide and the following results obtained:

C = 6.25; H = 0.81; P = 0.95; Ba = 55.32 per cent.

The phosphorus was present in organic combination. The ash was found to consist principally of barium carbonate. When the substance was heated with concentrated sulphuric acid a gas was liberated which caused a white precipitate of barium carbonate when led into barium hydroxide solution.

Judging by the analysis and reactions the crystalline substance was an impure barium oxalate, mixed with some of the barium salt of the organic phosphoric acid.

That the substance was barium oxalate was further confirmed by the following experiments:

It was re-crystallized several times from hot dilute hydrochloric acid by partially neutralizing with ammonia. It was then transformed into the silver salt as follows: the barium oxalate was dissolved in a little hot dilute nitric acid, diluted with water and silver nitrate added which caused a heavy white granular precipitate. This was filtered off, washed in water, alcohol and ether and dried in the air. The substance showed all the properties of silver oxalate, viz., it was very insoluble in dilute nitric acid and on heating the dry substance it exploded. It was, however, not free from phosphorus. It contained 68.41 per cent of silver while silver oxalate should contain 71.05 per cent of silver.

The balance of the barium oxalate was then transformed into calcium oxalate and the latter recrystallized many times from boiling dilute hydro-

chloric acid by nearly neutralizing with ammonia and acidifying with acetic acid. After purifying in this way 0.0826 gram of the substance was burned to constant weight in a platinum crucible. The calcium oxide remaining weighed 0.0310 gram. Calculated for the above quantity of $\text{CaC}_2\text{O}_4 + \text{H}_2\text{O} = 0.0316$ gram CaO .

The calcium oxide was dissolved in dilute nitric acid and tested with ammonium molybdate. A faint precipitate separated showing that some phosphorus still remained. There appears, however, to be no doubt that the substance was a nearly pure calcium oxalate.

Preparation of the silver salt of the organic phosphoric acid.

The water-soluble barium salt, analyzed as reported on page 432, was transformed into the silver salt as follows: 5 grams of the barium salt were suspended in water and the barium precipitated with slight excess of dilute sulphuric acid. It was filtered and the filtrate neutralized with ammonia. Silver nitrate was added producing a pure white precipitate which, however, rapidly darkened in color. It was filtered, washed in water and dried in vacuum over sulphuric acid under exclusion of light. The substance was then a heavy dark-gray amorphous powder. It was free from all but traces of ammonia and it did not contain any inorganic phosphate. For analysis it was dried at 105° in vacuum over phosphorous pentoxide.

Found: C = 9.49; H = 1.48; P = 7.89; Ag = 54.85 per cent.

As will be noticed it corresponds very closely in composition to the barium salt from which it was prepared and not to the compounds analyzed by Rather.

Preparation of the crude acid from wheat bran by the method of Rather.

In the preparation of the acid, the directions of the above author were followed as closely as possible. The various operations may be briefly stated as follows:

Wheat bran was digested in 0.2 per cent hydrochloric acid for three hours with frequent stirring. It was then strained through cheese-cloth, the residue was washed with water and again strained. The extract was centrifugalized and finally filtered. Copper acetate solution was added in

excess and allowed to stand over night. The copper precipitate was freed from the mother-liquor as far as possible by the centrifuge and finally washed with water repeatedly on the Buchner funnel. It was then suspended in water and decomposed with hydrogen sulphide, filtered and the filtrate evaporated to a thin syrup on the water bath. It was then dissolved in a small quantity of water and rendered strongly alkaline with ammonia and allowed to stand for twenty-four hours. The precipitate was filtered off and the filtrate evaporated on the water-bath until the excess of ammonia was driven off. It was then diluted with water and precipitated with barium chloride in excess, filtered, and washed in water, suspended in water and the barium precipitated with a slight excess of sulphuric acid. After filtering, the filtrate was neutralized with ammonia and again precipitated with barium chloride. These operations were repeated three times. After finally removing the barium, the acid filtrate was precipitated with copper acetate. The copper precipitate was filtered and washed with water until the filtrate gave no reaction with barium chloride. It was then suspended in water and decomposed with hydrogen sulphide and the filtrate evaporated on the water-bath to a syrupy consistency. This syrup was poured into 1600 cc. of alcohol and allowed to stand for the precipitate to settle. It was then filtered and again evaporated on the water-bath until the alcohol was removed. The residue was the crude acid which was obtained as a brown colored syrup. It was diluted with water to 100 cc. in which it gave a slightly opalescent solution of faint aromatic odor. It was divided into two parts; 75 cc. were used for the preparation of the barium salt; of the balance 10 cc. were used for the preparation of the silver salt.

Preparation of the silver salt from the crude acid.

Ten cc. of the above acid solution were diluted to 100 cc. with water and ammonia added to alkaline reaction. The excess of ammonia was boiled off, the solution cooled and silver nitrate added which caused a voluminous yellow-colored precipitate. It was filtered, washed in water and dried in vacuum over sulphuric acid under exclusion of light. It was then obtained as a very dark-colored heavy amorphous powder. The filtrate from the above was quite acid in reaction. It was neutralized with ammonia when a further quantity of a yellowish precipitate came down. More silver nitrate was added and the precipitate filtered, washed and dried as before. This was also obtained as a dark-colored amorphous powder. These precipitates were free from all but traces of ammonia. These silver precipitates were analyzed after drying at 105° in vacuum over phosphorus pentoxide.

The first precipitate gave:

C = 2.18; H = 0.53; Ag = 71.82; Total phosphorus = 7.82; Inorganic phosphorus = 5.61 per cent.

The inorganic phosphorus was determined as follows:

The substance was suspended in cold water and dissolved by the addition of cold dilute nitric acid and the silver precipitated with hydrochloric acid. The filtrate was neutralized with ammonia, acidified with nitric acid, ammonium nitrate and ammonium molybdate added and the whole kept at a temperature of 65° for one-half hour. The precipitate was then filtered and the phosphorus determined as magnesium pyrophosphate as usual.

The composition of the above silver precipitate is quite different from that reported by Rather. It will be noticed, however, that the inorganic phosphorus is equivalent to 75.64 per cent of inorganic silver phosphate.

The second precipitate gave:

C = 0.98; H = 0.37; Ag = 74.37; total phosphorus = 7.15; Inorganic phosphorus = 6.73 per cent. This substance accordingly contained 90.74 per cent of inorganic silver phosphate.

Preparation of the barium salt from the crude acid.

Seventy-five cc. of the crude acid were diluted to 500 cc. with water and precipitated with barium hydroxide in excess. The resulting barium precipitate was reprecipitated as before alternately four times with barium hydroxide and five times with alcohol from 0.5 per cent hydrochloric acid. After finally filtering, washing in dilute alcohol, alcohol and ether and drying in vacuum over sulphuric acid, 7 grams of a snow-white amorphous powder were obtained. It was free from chlorides and inorganic phosphate. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

Found: C = 12.31; H = 2.21; P = 13.99; Ba = 33.45 per cent.

It will be noticed that, after removing inorganic phosphate, the composition of the barium salt does not agree with a compound of the formula $C_{12}H_{41}O_{42}P_9$, as proposed by Rather but that the composition agrees closely with the compound $C_{20}H_{45}O_{49}P_9Ba_5$ which we described in an earlier paper (*loc. cit.*).

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Calculated for the above:

C = 11.79; H = 2.21; P = 13.71; Ba = 33.76 per cent.

A second lot of the acid was prepared from wheat bran by the same method as before except that the various concentrations were done in vacuum at a temperature of 40°–45° and not on the water-bath as the first time.

The silver and barium salts were prepared from the crude acid exactly as before.

The silver precipitate gave the following results after drying at 105° as before:

Found: C = 4.56; H = 0.77; Ag = 65.88; total phosphorus = 8.03; inorganic phosphorus = 3.15 per cent.

This approaches in composition the precipitates analyzed by Rather. However, as shown by the above percentage of inorganic phosphorus it contained 42.54 per cent of inorganic silver phosphate.

The barium precipitate was free from chlorides and inorganic phosphate. It was analyzed after drying at 105° as before.

Found: C = 11.66; H = 2.11; P = 14.14; Ba = 34.36 per cent.

This substance is therefore identical in composition with the first preparation reported above and it agrees very closely with the compounds previously described.

It is clearly evident from the result recorded above that an acid, $C_{12}H_{41}O_{42}P_9$, such as described by Rather, does not exist, at least not in wheat bran or cottonseed meal. The alleged pure, homogeneous silver salts analyzed by the above author must have been largely contaminated with silver phosphate and this simple impurity escaped his observation.

Further preparations of barium salts of the organic phosphorus compound of wheat bran.

The 0.2 per cent hydrochloric acid extract of wheat bran was precipitated by adding a concentrated solution of barium chloride. After settling, the precipitate was filtered and washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid.

To the mother-liquor from above, barium acetate was added which caused a further precipitate. This was filtered, washed and dried as above.

Total and inorganic phosphorus were determined in these precipitates as follows: Total phosphorus was determined after decomposing by the Neumann method. As inorganic phosphorus we consider the amount of phosphorus directly precipitated by ammonium molybdate from the nitric acid solution of the substance at a temperature of 65° for one-half hour.

Found in the precipitate produced with barium chloride:

Total phosphorus = 8.45 per cent; Inorganic phosphorus = 1.55 per cent.

The result shows that 18.32 per cent of the total phosphorus was present as inorganic.

Found in the precipitate produced with barium acetate:

Total phosphorus = 10.28 per cent; Inorganic phosphorus = 8.63 per cent.

In this precipitate 83.96 per cent of the total phosphorus was present as inorganic.

The above barium precipitates were purified separately in the same way as before by repeatedly precipitating with barium hydroxide and alcohol alternately from 0.5 per cent hydrochloric acid until pure white amorphous powders were obtained which were free from inorganic phosphate and which contained no bases other than barium. After drying at 105° in vacuum over phosphorus pentoxide the following results were obtained on analysis: The preparation isolated from the barium chloride precipitate gave:

C = 11.53; H = 2.10; P = 14.29; Ba = 34.60 per cent.

This substance has the same composition as the precipitates obtained from the previously isolated crude acid. These various preparations were therefore mixed and treated as will be described later. The preparation isolated from the barium acetate precipitate gave:

C = 15.22; H = 2.62; P = 12.22; Ba = 29.85 per cent.

Several other preparations were made from wheat bran in different ways. The composition varied considerably, however, as is evident from the figures given below.

- I. C = 15.33; H = 2.70; P = 11.61; Ba = 32.31 per cent.
- II. C = 14.62; H = 2.65; P = 12.84; Ba = 30.59 per cent.
- III. C = 13.94; H = 2.47; P = 13.10; Ba = 31.41 per cent.
- IV. C = 15.01; H = 2.67; P = 12.25; Ba = 30.94 per cent.

Treatment of the amorphous barium salt with cold water.

The barium precipitates obtained from the crude acid as well as the substance isolated from the barium chloride precipitate which, as shown above, all had the same composition were mixed together, total weight 22.5 grams, and rubbed up in a mortar with 200 cc. of cold water in which the greater portion dissolved. The insoluble substance was filtered, washed in water, alcohol and ether and dried in vacuum over sulphuric acid. It was then treated with a second portion of 200 cc. of water, again filtered, washed and dried. This insoluble residue weighed 5.75 grams. It had the following composition after drying at 105° as before:

Found: C = 9.52; H = 1.53; P = 12.89; Ba = 41.79 per cent.

The filtrate from the above, containing the water-soluble portion of the barium salt, was heated to boiling. The solution turned first cloudy but gradually a white flocculent precipitate separated which soon assumed a granular form and settled to the bottom of the flask. This was filtered, washed in boiling water, alcohol and ether and dried in the air. Under the microscope the substance showed no definite crystalline structure but consisted of fine transparent globules. The preparation was free from inorganic phosphate. It weighed 1.6 grams. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide:

Found: I. C = 9.15; H = 1.79; P = 13.57; Ba = 40.43; H₂O = 9.53 per cent.

II. C = 8.96; H = 1.62 per cent.

The filtrate from the above was first neutralized with barium hydroxide. The white amorphous precipitate was filtered, washed in water, alcohol and ether and dried in vacuum over

sulphuric acid. It was free from inorganic phosphate and weighed 17 grams. It was analyzed after drying at 105° as above.

Found: C = 9.19; H = 1.42; P = 11.21; Ba = 47.86 per cent.

Crystallization of the water-insoluble barium salt.

The water-insoluble barium salt, 5.75 grams, previously analyzed, was dissolved in the least possible quantity of 0.5 per cent hydrochloric acid and the solution heated to boiling. The solution remained perfectly clear. Alcohol was then added until the solution turned slightly cloudy. On scratching, the substance began to separate in a crystalline or granular form on the sides of the flask.

After standing over night, the substance was filtered, washed in water, alcohol and ether and dried in the air. The filtrate was precipitated with alcohol, filtered, washed and dried in vacuum over sulphuric acid. It was again dissolved in a small quantity of 0.5 per cent hydrochloric acid, heated, alcohol added and allowed to stand as before when a further quantity of the same shaped crystalline or granular product was obtained. This was filtered, washed in water, alcohol and ether and dried in the air. The substance was a heavy snow-white crystalline or granular powder. Under the microscope it looked homogeneous and consisted of small transparent globules. It was free from chlorides and inorganic phosphate. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

Found: C = 9.91; H = 1.83; P = 14.90; Ba = 34.75; H_2O = 12.48 per cent.

This substance was recrystallized in the same manner when it separated in the same form as before. It was again analyzed after drying as 105° as above.

Found: C = 10.06; H = 2.02; P = 15.30; Ba = 33.36; H_2O = 11.73 per cent.

Since this substance separated in characteristic crystalline manner and did not show any appreciable change in composition on recrystallization one might believe that it was homogeneous. We have, however, been unable to obtain any other preparation having the same composition. Numerous preparations were made which separated in exactly the same manner and form and

so far as appearance is concerned they looked identical but on analysis widely varying results were obtained.

One product gave:

C = 10.67; H = 2.00; P = 14.46; Ba = 35.02; H₂O = 11.97 per cent.

Another after recrystallizing three times gave the following:

C = 11.60; H = 1.88; P = 12.48; Ba = 34.37; H₂O = 11.60 per cent.

A third preparation gave:

C = 11.26; H = 1.58; P = 10.67; Ba = 37.81; H₂O = 12.00 per cent.

It would seem evident from the results reported that wheat bran contains more than one organic phosphoric acid. It appears probable that several such acids are present and that the solubility of the salts of these acids differs so slightly that their separation is very difficult. Until definitely homogeneous products can be separated from this mixture it seems futile to develop empirical formulas; for such can be calculated for every substance analyzed. The investigation is being continued.

The author wishes to express his appreciation and thanks to Dr. P. A. Levene of the Rockefeller Institute for Medical Research, New York, N. Y. and to Dr. Thomas B. Osborne of the Connecticut Agricultural Experiment Station, New Haven, Conn. for many valuable suggestions.

CONCERNING THE ORGANIC PHOSPHORIC ACID COMPOUND OF WHEAT BRAN. III.

INOSITE MONOPHOSPHATE, A NEW ORGANIC PHOSPHORIC ACID OCCURRING IN WHEAT BRAN.

TENTH PAPER ON PHYTIN.

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(Received for publication, June 10, 1914.)

INTRODUCTION.

In previous reports¹ we have shown that the crude organic phosphorus compound of wheat bran² can be separated into two portions by treating it with barium hydroxide. The insoluble precipitate which forms under these conditions contains the barium salts of certain not yet identified organic phosphoric acids and it is free from nitrogen. By evaporating the filtrate from the above insoluble barium salts a substance is obtained which is rich in nitrogen and which also contains phosphorus in organic combination.

In the further investigation of this soluble, nitrogen-containing substance it was found that its aqueous solution gave an insoluble precipitate with lead acetate. The only other salt which gave any

¹ This *Journal*, xii, p. 447, 1912; N. Y. Agric. Exp. Station Tech. Bull. 22, 1912; also preceding article.

² This crude compound had been prepared by precipitating the 0.2 per cent hydrochloric acid extract of wheat bran with alcohol. The resulting precipitate was then purified by repeatedly precipitating from 0.2 per cent hydrochloric acid with alcohol until a nearly white product was obtained which was easily soluble in cold water and which gave no precipitate with ammonium molybdate. For particulars concerning its preparation see the above publications.

precipitate was copper acetate and then only on warming, when a bluish-white amorphous precipitate was produced which dissolved completely on cooling.

The aqueous solution was therefore treated with lead acetate in excess. The resulting precipitate was filtered, washed and decomposed with hydrogen sulphide. These operations were repeated several times until a perfectly white lead precipitate was obtained. This was finally decomposed with hydrogen sulphide and the solution concentrated in vacuum until a thick, practically colorless syrup remained. On scratching with a glass rod this immediately crystallized to a white solid mass. On recrystallizing the substance from water with the addition of alcohol it was obtained in beautiful colorless star-shaped aggregates of plates or long prisms. When its aqueous solution is slowly concentrated it crystallizes in large colorless prisms with pointed ends, being often arranged in star-shaped bundles. It is, however, so soluble in water that it is more expedient to crystallize it from water with addition of alcohol.

The substance was free from bases and it was also free from nitrogen and sulphur but it contained phosphorus in organic combination. Analysis showed that it was inosite monophosphate, $C_6H_{13}O_9P$ or $C_6H_6(OH)_5O.PO(OH)_2$. On cleavage either with dilute sulphuric acid at 120° or higher or with 10 per cent ammonia at 150° in a sealed tube it decomposes into inosite and phosphoric acid.

So far as we are aware inosite monophosphate has not been known previously and we believe that this is the first time that it has been isolated. It is interesting to note in connection with the "phytin problem" that a compound like inosite monophosphate exists in nature. Clarke³ in a recent paper reports the isolation from wild Indian mustards of certain crystalline strychnine salts of what he believes to be inosite tetra- and diphosphate in addition to inosite hexaphosphate. It appears probable therefore that in certain plants the organic phosphoric acids may be present not only as phytic acid or inosite hexaphosphate, $C_6H_{18}O_{24}P_6$, but also as lower phosphoric acid esters of inosite. From wheat bran, for instance, we have been unable to isolate any inosite hexa-

³ *Journ. Chem. Soc.*, cv, p. 535, 1914.

phosphate. The insoluble barium salts of the organic phosphorus compound from this material are evidently mixtures of various organic phosphoric acids, either lower inosite phosphates or phosphoric acid esters of other carbohydrates. However, we have been unable, so far, to separate any homogeneous substance from this mixture.

The isolation of inosite monophosphate only succeeded because its properties are so different from the other organic phosphoric acids which exist in wheat bran; for instance, its easily soluble barium salt permitted its separation from the other acids which give insoluble barium salts.

At present we have no data as to the quantitative percentage of inosite monophosphate in wheat bran. We hope, however, to make some determinations in this direction later. We wish to reserve the further study of the physiological properties of this substance in connection with the general investigation which is being carried out at this station. We also beg to reserve the study of the cleavage products obtained under different conditions and other derivatives of inosite monophosphate.

EXPERIMENTAL PART.

The crude nitrogen-containing substance⁴ was dissolved in water and a concentrated solution of lead acetate added in excess. The resulting precipitate was filtered, washed thoroughly in cold water and then suspended in hot water and decomposed with hydrogen sulphide. The lead sulphide was filtered off and the filtrate boiled to expel hydrogen sulphide. The solution was then of strongly acid reaction and it had a sharp acid taste. It was again precipitated as above three times with lead acetate. The pure white colored lead precipitate which was finally obtained was decomposed with hydrogen sulphide. The filtrate was concentrated in vacuum at a temperature of 40°–45° and then dried in vacuum over sulphuric acid until a thick practically colorless syrup remained. On scratching with a glass rod this immediately began to crystallize forming a white solid mass. It was very

⁴ For its isolation from wheat bran see this *Journal*, xii, p. 456, and *Tech. Bull.* 22, p. 10, N. Y. Agric. Exp. Station, 1912; also the preceding article.

soluble in water but insoluble in alcohol. It was extracted several times with 95 per cent alcohol, filtered and washed in absolute alcohol and ether and dried in the air. For recrystallization it was dissolved in a small quantity of water and absolute alcohol added until the solution turned slightly cloudy. On scratching, the substance began to crystallize. After standing in the ice chest over night it had separated in large colorless plates or prisms arranged in star-shaped aggregates. It was recrystallized a second time in the same manner.

The substance was free from bases and also free from nitrogen and sulphur but it contained phosphorus in organic combination. The aqueous solution gave no precipitate with ammonium molybdate on being kept at a temperature of 65° for some time but after decomposing by the Neumann method it gave a heavy precipitate of ammonium phosphomolybdate with this reagent.

The substance has no sharp melting point. When rapidly heated in a capillary tube it softens at 200° and decomposes under effervescence at 201° – 202° ; when slowly heated it begins to soften at 188° and melts under decomposition at 190° – 191° (uncorrected). It is optically inactive. A 10 per cent solution in a 1 dm. tube shows no rotation.

For analysis it was dried at 100° in vacuum over phosphorus pentoxide but it did not lose in weight. 0.1550 gram substance gave 0.0749 gram H_2O and 0.1566 gram CO_2 . 0.0766 gram substance gave 0.0325 gram $\text{Mg}_2\text{P}_2\text{O}_7$.

Found: C = 27.55; H = 5.40; P = 11.82 per cent.

For inosite monophosphate, $\text{C}_6\text{H}_{11}\text{O}_9\text{P}$ = 260.

Calculated: C = 27.69; H = 5.00; P = 11.92 per cent.

Titrated against barium hydroxide, using phenolphthalein as indicator, it forms the neutral barium salt, $\text{C}_6\text{H}_{11}\text{O}_9\text{P Ba}$.

0.1985 gram substance required 7.6 cc. $\frac{N}{5}$ $\text{Ba}(\text{OH})_2$.

For $\text{C}_6\text{H}_{11}\text{O}_9\text{P Ba}$ calculated 7.6 cc. $\frac{N}{5}$ $\text{Ba}(\text{OH})_2$.

Properties of inosite monophosphate.

The substance is very soluble in water. The aqueous solution shows a strong acid reaction to litmus and it has a sharp, somewhat astringent acid taste. It is insoluble in alcohol, ether and in the other usual organic solvents. Its aqueous solution gives

no precipitate with barium hydroxide or with calcium or barium chlorides; Ammonia produces no precipitate in these solutions but the addition of alcohol causes white amorphous precipitates. Silver nitrate produces no precipitate even in a solution neutralized with ammonia. When alcohol is added to the solution containing silver nitrate a white amorphous precipitate is produced which dissolves on warming; on cooling the silver salt separates in small round crystal aggregates. It gives no precipitate with ferric or mercuric chloride nor with copper sulphate. In the cold no precipitate is produced with copper acetate but on warming this solution a bluish-white precipitate separates which again dissolves completely on cooling. With excess of lead acetate a heavy white amorphous precipitate is formed which is but slightly soluble in dilute acetic acid but readily soluble in dilute hydrochloric or nitric acid. Ammonium molybdate produces no precipitate in either dilute or concentrated aqueous solutions.

The acid crystallizes without water of crystallization from either water or dilute alcohol.

Cleavage of inosite monophosphate into inosite and phosphoric acid.

I. *Acid hydrolysis.* The acid, 0.35 gram, was heated in a sealed tube with 15 cc. of 3 per cent sulphuric acid to 120°-125° for about three and one-half hours. After cooling, the liquid was of a pale straw color. The sulphuric and phosphoric acids were precipitated with barium hydroxide and the excess of barium removed with carbon dioxide. The filtrate was evaporated to dryness on the water-bath. The residue gave no precipitate with ammonium molybdate but after decomposing by the Neumann method a heavy yellow precipitate was produced with this reagent showing that only a portion of the acid has been hydrolyzed under the above condition. The residue, however, contained some inosite which was isolated as follows: The substance was taken up in a little water, somewhat more than an equal volume of alcohol was added which caused a voluminous white amorphous precipitate consisting of the unchanged barium salt of inosite monophosphate. After filtering, the precipitate was again dissolved in water, again precipitated with alcohol and filtered. The filtrates were evaporated on the water-bath, taken up in a little hot water and the inosite brought to crystallization by the

addition of alcohol and ether. It separated in the usual needle-shaped crystals. After standing several hours in the ice chest, the crystals were filtered, washed in alcohol and ether and dried in the air. Yield 0.06 gram. It gave the reaction of Scherer and melted at 224° (uncorrected).

II. *Alkaline hydrolysis.* Another portion of the acid, 0.4 gram, was heated in a sealed tube with 10 cc. of 10 per cent ammonia for six hours to 120° . The solution then contained some free phosphoric acid as it gave a precipitate with ammonium molybdate but the greater portion of the substance remained unchanged. It was impossible to isolate any inosite from this reaction mixture. After precipitating the barium with sulphuric acid, the residue was again heated in a sealed tube with 10 per cent ammonia for about four and one-half hours to 150° . In this case complete hydrolysis had taken place and after isolating the inosite in the usual way 0.15 gram was obtained. It was recrystallized four times from dilute alcohol with addition of ether and was then obtained in colorless needles free from water of crystallization. It gave the reaction of Scherer and melted at 224° (uncorrected). The identity of the substance was further confirmed by the analysis.

0.1206 gram substance gave 0.0752 gram. H_2O and 0.1761 gram CO_2 .

Found: C = 39.82; H = 6.97 per cent.

For $\text{C}_6\text{H}_{12}\text{O}_6 = 180$.

Calculated: C = 40.00; H = 6.66 per cent.

SUMMARY.

A previously unknown organic phosphoric acid, inosite monophosphate, $\text{C}_6\text{H}_{13}\text{O}_9\text{P}$, has been isolated in beautiful crystalline form from wheat bran. All of the salts of this acid, with the exception of the lead salt, are very soluble in cold water. The alkaline earth salts are not precipitated with ammonium hydroxide, differing in this respect from other known organic phosphoric acids as well as from ordinary phosphoric acid.

The author wishes to express his appreciation and thanks to his Excellence, Prof. E. Fischer and to Prof. H. Leuchs for the kind interest which they have shown in the work reported in this paper.

THE BEHAVIOR OF THE SUGAR AND LACTIC ACID IN THE BLOOD FLOWING FROM THE LIVER, AFTER TEMPORARY OCCLUSION OF THE HEPATIC PEDICLE.

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The significance of lactic acid in the intermediary metabolism of carbohydrates has become more and more evident within recent years. Without going into details at present, it may however be well to point out that it has been shown that there are at least three main metabolic processes from which lactic acid may be derived. Hopkins and Fletcher¹ have demonstrated the production of lactic acid in surviving muscle when there is a limited supply of oxygen, and Embden and his pupils² have found it to accumulate under certain conditions in expressed muscle juice. Ryffel³ has found that it appears in relatively large amount in the urine after such strenuous muscular exercises that tissue respiration is inadequate. The source of this lactic acid is not definitely carbohydrate. Embden and Kraus,⁴ and Oppenheim⁵ have found lactic acid to be produced in large amount when the dog liver is artificially perfused with ox blood, provided either that the liver contain glycogen or that the perfusion fluid contain dextrose, di-oxyacetone, glycerinaldehyde, glycerine or *dl*-alanine.

¹ Hopkins and Fletcher: *Journ. of Physiol.*, xxxv, p. 247, 1906; Fletcher: *ibid.*, xliii, p. 286.

² Embden, Kalberlah and Engel: *Biochem. Zeitschr.*, xlv, p. 45, 1912.

³ Ryffel: *Journ. of Physiol.*, xxxix, 1909 (Proc. Physiol. Soc.)

⁴ Embden and Kraus: *Biochem. Zeitschr.*, xlv., p. 1., 1912; see also Embden, Schmitz and Wittenberg: *Zeitschr. f. physiol. Chem.*, lxxxviii, p. 210, 1913.

⁵ Oppenheim: *ibid.*, p. 30.

Although the chief source of the lactic acid in these experiments is, evidently carbohydrate, yet it is significant that amino-acids may also produce it.

During the process of glycolysis in blood, lactic acid becomes produced as dextrose disappears, and it is particularly by observations on this process that accurate knowledge is accumulating concerning the nature of the chemical process through which the breakdown of dextrose occurs.⁶

These studies have prompted us to undertake an investigation of the conditions which control the appearance of lactic acid in the living animal, and the following paper is a brief review of some of the results which so far we have obtained.

The investigations were made on blood collected from the vena cava opposite the hepatic veins after ligating the vein below and temporarily occluding it in the thorax. The method used for analysis was practically the same as that of von Fürth and Charnass.⁷ Instead of reducing the volume of the protein- and mercury-free filtrate by evaporation *in vacuo*, it was placed in a photographic dish and a stream of moderately warmed air blown over it. Lind's admirable apparatus was used for the ether extraction, and after removal of the ether, the lactic acid was oxidized by $\frac{N}{10}$ permanganate, and the resulting acetaldehyde titrated with bisulphite. Duplicate analyses were made whenever possible, and these have agreed satisfactorily.

The particular problem which we have investigated is the production of lactic acid following a disturbance in the blood supply of the liver. For this purpose a ligature was placed loosely around the portal vein, and in most experiments the hepatic artery was also ligated. A sample of blood having been collected as above described, and the thoracic ligature around the vena cava loosened, the portal vein and usually the hepatic artery were occluded for varying periods up to five minutes, immediately after which another sample of blood was collected. The circulation was then reëstablished, and in most of the experiments a third sample of

⁶ Levene and Meyer: this *Journal*, xi, p. 361, 1912; Kraske: *Biochem. Zeitschr.*, xlv, p. 81, 1912; Kondo: *ibid.*, p. 88; von Noorden, J.: *ibid.*, p. 94; Loeb, A.: *ibid.*, 1, p. 451, 1913; Griesbach: *ibid.*, p. 457.

⁷ Cf. Embden: *Abderhalden's Handbuch der biochemischen Arbeitsmethoden*, v, p. 1254, 1912.

blood removed after about fifteen minutes. Further details are given in the table.

Concerning the amount of sugar in the liver blood, it will be noted that this is subject to wide variations even in the so-called normal samples which were collected immediately after the preliminary operations. The minimum was 0.128 per cent, and the maximum 0.355. Although it is true that the blood containing the former amount was from a starved dog, and that the higher percentages were usually found in the blood of sugar-fed animals, yet no constant relationship between the sugar content of the diet and that of the liver blood can be made out. Local conditions having temporary influence on the glycogenolytic process in the liver evidently count for more in determining the discharge of sugar into the hepatic veins than the amount of glycogen which may happen to be present in the liver cells. Since these "normal" samples of blood were removed just after the application of the thoracic ligature around the vena cava, it may be that the temporary asphyxia which this operation caused is responsible for the hyperglycogenolysis. This will be more thoroughly investigated by us in the near future.

Notwithstanding this irregularity in the normal values, a very distinct increase in sugar concentration was found present in the blood collected immediately after the removal of the clamp from the hepatic pedicle, and in blood removed in from five to fifteen minutes later, this increase was still more marked, except in the case of the starved dog, where it had disappeared. There are, no doubt, two causes for the increase on sugar: (1) The stagnation of blood so that sugar accumulated in it, and (2) local asphyxia of the liver cell. The fact that after fifteen minutes of normal blood flow, the sugar usually continued to mount in the blood points to something more than mere stagnation of blood as the cause of the increase in sugar; it shows us that an asphyxial hyperglycogenolysis has been set up.

Coming to the lactic acid, we find that the normal percentage varies between 0.0218 and 0.09, this latter figure, however, being for some unexplainable reason far above that of the majority of cases, which do not mount above 0.05. The average of ten independent observations (exclusive of that in Exp. 24) is 0.033, seven of them lying between 0.022 and 0.037. The true normal

The percentage of sugar and lactic acid in the blood flowing from the liver before and after temporary occlusion of the blood flow.

EXP.	EXPERIMENTAL CONDITION	TIME	DEXROSE	LACTIC ACID	REMARKS
			<i>per cent</i>	<i>per cent</i>	
13	Normal		0.239	0.051	Sugar-fed.
			.	0.049	
14	Normal		0.260	0.023	Not sugar-fed.
15	Normal		0.176	0.0244	Not sugar-fed.
				0.0192	
17	Portal vein occasionally clamped during period of 5 min. Hepatic art. patent.	10.45 Clamp 10.50	0.355 0.276	0.0483 0.0476	Sugar-fed. Estimation of dextrose uncertain because copper nearly all reduced.
18	Portal vein occasionally clamped for 2 min. at a time. Hepatic art. patent during most of the period.	10.30 Clamp 10.45	0.323 0.497	0.0326 0.0422	Sugar-fed. Kept for some days before distilling.
				0.0594	Fall in B. P. slight.
19	Same as 18, only hepatic art. ligated throughout.	10.30 Clamp 10.45	0.103 Lost	0.026 0.064	Sugar fed. Blood very venous. Large excess of blood taken. Fall in B. P. marked.
20	Clamp on both vessels for 2 min.	Normal Clamp 2 min. 5 min. later	0.209 0.255 0.320	0.0224 0.0406 0.0523	Not sugar-fed. (From now on Schenck method also used for sugar.) Fall in B. P. marked.
21	Same as 20	Normal Clamp 2 min. 5 min. later	0.201 0.227 0.294	0.0243 0.0587 0.0612	Sugar-fed. Fall in B. P. slight.

EXP.	EXPERIMENTAL CONDITION	TIME	DEXTROSE	LACTIC ACID	REMARKS
			<i>per cent</i>	<i>per cent</i>	
22	Clamp on both vessels for 5 min. Occlu- sion possibly only partial	Normal Clamp 2 min. 5 min. later	0.135 0.104 0.140	0.0346 0.0247 0.0406	Sugar fed. Perhaps slightly alkaline. Fall in B. P. slight and viscera not engorged. Not certain that clamp properly applied.
23	Same as 22 but on starved dog.	Normal Clamp 5 min. 15 min. later	0.128 0.226 0.107	0.0429 0.0567 0.0758	Starved 2 days. Ether extraction 48 hrs. Fall in B. P. marked. Artificial respi- ration neces- sary after re- moving clamp.
24	Same as 22	Normal Clamp 5 min. 15 min. later	0.327 0.638+ 0.638+	0.090 0.100 0.120	Sugar-fed. Ether extraction 48 hrs. + more t h a n t h e amount. Fall in B. P. marked. Blood intensely venous.

value may possibly be somewhat higher than this, for we have latterly found that a forty-eight-hour extraction with ether in Lind's apparatus probably removes a small amount more of lactic acid from the protein-free blood filtrate than is removed in twenty-four hours, which was the duration of extraction in most of our observations. The excess thus removed must, however, be very small, as Ishihara has found.⁸

As with the sugar content, it is impossible, in these experiments, to make out any definite relationship between the probable glycogen content of the liver and the amount of lactic acid in the liver

⁸ Ishihara: *Biochem. Zeitschr.*, 1, p. 468. 1913.

blood. It will be necessary to investigate this point more closely, for in the present investigation it was impossible for various reasons to give it special attention.

The clamping experiments were of three varieties. In experiments 17 and 18 the portal vein alone was clamped, and this for two minutes time with normal intervals of corresponding length. The percentage of lactic acid remained unchanged after five minutes in one experiment, but distinctly increased after fifteen minutes in the other.

In experiments 19, 20 and 21, besides the portal vein, the hepatic artery was occluded with the result that after two minutes the lactic acid concentration was at least doubled, and this even in so short a time as two minutes after the application of the clamp (No. 20). In five minutes after the removal of the clamp, the lactic acid, like the sugar, was still higher than immediately after its removal, although the increase was comparatively small as compared with the previous one.

The third group includes experiments 23 and 26 (No. 22 is omitted in this classification because of uncertainty as to certain details), in which a clamp was applied to the portal vein for five minutes, the hepatic artery being permanently ligated. Although for unknown reasons, the initial percentage of lactic acid was unusually high in both these experiments, a distinct increase is seen to have occurred in them both, and to have been continued for fifteen minutes after removal of the clamps.

These experiments demonstrate clearly that lactic acid is readily produced in the liver as a result of local stagnation of blood flow. This production of lactic acid is probably dependent upon the asphyxial condition which is induced, and it is very likely from glycogen that it is derived. This may not be its only source, but it is at least significant that our observations, with one exception (No. 23), the percentages of sugar and of lactic acid have run more or less parallel.

Having thus orientated ourselves as to its general behavior, we intend proceeding at once with a further investigation of this problem, especially with regard to the possibility that in those conditions in which glycogen rapidly disappears from the liver, without any marked degree of hyperglycaemia becoming established, it does so by becoming, in part at least, converted to lactic acid.

ON SPHINGOMYELIN.

SECOND PAPER.¹

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(Received for publication, June 16, 1914.)

Sphingomyelin was first discovered by Thudichum² and later studied by Rosenheim and Tebb.³ Thudichum was also the first to have accomplished a hydrolysis of the substance, and to have attempted the identification of its components. As such he described two basic substances; sphingosine and neurine; an acid, sphingostearic acid; an alcohol, sphingol, and phosphoric acid. Thudichum based his conclusions on analytical data. The data and conclusions will be discussed later in connection with the individual components. Rosenheim and Tebb have improved the method of preparation of the substance. Regarding the components of sphingomyelin these authors refer only to the bases and to the alcohol. They speak of the bases as choline and sphingosine but do not substantiate their conclusions by analytical data, or by any other experimental evidence.

The ultimate aim of the present work was to establish the structure of sphingomyelin. As introductory to this an attempt was made to discover the conditions which would make possible the preparation of a pure sphingomyelin, and further, to establish conditions of hydrolysis leading to a satisfactory yield of its principal components.

The aim of the preliminary work in a measure was accomplished. The conditions were found which made possible the preparation of the phosphatide entirely free from galactosides. The conditions

¹ This *Journal*, xv, p. 153, 1913.

² Thudichum: *On the Chemical Constitution of the Brain*, London, 1884.

³ Rosenheim and Tebb: *Quart. Journ. of Physiol.*, i, p. 297, 1908; *Journ. of Physiol.*, xli, *Proc. Physiol. Soc.*, July 9, 1910.

were also found which permitted a satisfactory hydrolysis, so that every component could be obtained in quantities permitting their purification. Since the present work principally aimed to establish methods of analysis, it was carried out on material of only relative purity. Further purification is accompanied with great loss of material and time, and the presence of a little impurity does not affect much the course of hydrolysis and the yields of the principal components. On the other hand, the results of the analysis of even slightly impure material does not permit of a final conclusion regarding the number and the character of the components of sphingomyelin. In fact, the results of the present hydrolysis raise the question whether or not there exist more than one sphingomyelin, or whether the substance has the structure of a polyphosphatide, of which every unit is composed of a different base and of a different organic acid.

Properties of sphingomyelin.

The substance was purified until it gave a negative test with orcin and copper, thus showing the absence in it of a carbohydrate. When obtained in that degree of purity the substance is optically inactive. It has the composition: C = 64; H = 11; N = 3.40; P = 3.60; inorganic bases = 3 per cent. The proportion of N : P = 2 : 1 (approximately). It contains no free amino-nitrogen in the molecule, and contains three methyl groups to two atoms of nitrogen, hence one nitrogen atom in the form of choline. The substance, dissolved in glacial acetic acid and alcohol, absorbs hydrogen.

Results of the hydrolysis of the substance. For hydrolysis a substance was employed that frequently showed a composition not much different from the purest material. The least purified material employed in the work was in its ultimate composition practically identical with the substance of Thudichum. This is seen from the following data:

TABLE I.

	C	H	N	P	O	INORGANIC BASES	METHOD OF PURIFICATION
Thudichum.....	65.37	11.29	2.96	3.24	17.04		
Present work:							
Sample 1.....	66.90	11.42	3.50	3.80		3.0	Pyridine and chloroform; negative orcin test.
Sample 2.....	66.90	11.12	3.37	3.73		4.5	Ligroin and alcohol; very faint orcin and copper test.
Sample 3.....	66.65	11.58	3.48	3.58		3.5	
Sample 4.....	67.60	11.71	3.64	3.36		0.0	
Sample 5.....	67.61	11.59	3.29	3.78		0.0	
Sample 6.....	67.44	11.76	3.29	3.69		0.0	
Sample 7.....	67.27	11.00	3.22	3.71		0.0	

On hydrolysis the following acid substances were obtained: Phosphoric acid, and two organic acids, cerebronic and lignoceric. It is noteworthy that the mixture of the two acids as obtained in our experiments had approximately the following composition: C = 76.5; H = 12.6 per cent. These figures are nearly identical with those obtained by Thudichum, and which caused him to regard the mixture as an isomeric stearic acid.

Of the basic substances one was identified without great difficulty—this was choline. On the other hand, it was found very difficult to obtain conclusive evidence regarding the character of the other base or of the other bases. A method of hydrolysis which was successfully employed in preparation of sphingosine from cerebrosides led to a substance with a constant composition. The analytical data of the sulphate and hydrochloride of the substance corresponded to the formula $C_{15}H_{31}NO_2$. However, this view soon was abandoned as erroneous. It was found that the base was contaminated with traces of phosphorus-containing bodies, although the total ash of the substance did not exceed 2 per cent. After much experimenting the conditions were discovered which permitted a complete hydrolysis of the original material and which at the same time left a reasonable proportion of the basic substances intact. However, the material obtained by this process had a varying composition. The base was always analyzed as the sulphate.

The carbon of this fluctuated between 62.5 to 63.5 per cent and the hydrogen between 10.3 to about 11.0 per cent. It was found convenient to reduce the bases with hydrogen and palladium before fractionation. Out of a chloroform-alcohol mixture it was possible to separate a sulphate having the following composition: C = 63.60-64; H = 11.5; N = 4.2-4; S = 4.4-4.9. A substance of this composition corresponds to a sulphate of a base having the composition: $(C_{17}H_{37}NO)_2 H_2SO_4$.

As yet we were never in possession of the material in a quantity sufficient for a satisfactory diagnosis of it. However, since the methods of preparation of both sphingomyelin, and its basic components are perfected, one may hope in the near future to reach a definite conclusion regarding the nature of the basic substances of sphingomyelin. Again on this occasion it is worthy of note that the basic substance obtained by Thudichum had an ultimate composition nearly identical with that of the fraction of the crude bases obtained in the present work. Thudichum regarded his substance as impure sphingosine. The present work favors rather the view that the fraction represents a mixture of two bases.

CONCLUSIONS.

The results of the present work permit of the following conclusions:

1. Sphingomyelin having the composition of the substance obtained by Thudichum is contaminated with galactosides.

2. It is not certain whether the sphingomyelin fraction of the brain lipoids, after it is freed from galactosides, contains only one phosphatide.

3. Sphingomyelin, apparently containing a very insignificant proportion of galactosides, is composed of the following substances: phosphoric acid, lignoceric and cerebronic acids, choline, sphingosine, and perhaps another base having the composition of $C_{17}H_{35}NO$. It remains to be established whether or not the latter substance is impure sphingosine, and if it is a different base, whether or not it is a primary constituent of sphingomyelin.

EXPERIMENTAL PART.

Preparation of sphingomyelin requires six principal stages:

1. Desiccated brain tissue is exhaustively extracted with boiling alcohol. Each extraction lasted about thirty minutes. On cooling this extract forms a precipitate.

2. The precipitate is exhaustively extracted with ether and acetone.

3. The residue is dissolved in hot technical pyridine and allowed to cool. On standing there forms a precipitate.

4. This precipitate is dissolved in hot glacial acetic acid and allowed to cool. There forms a precipitate. The mother liquor contains sphingomyelin. It is concentrated under diminished pressure and transferred into acetone. A precipitate forms consisting of crude sphingomyelin.

5. The substance obtained through treatment (4) is dissolved in 5 parts of ligroin and 1 of alcohol. An excess of 98 per cent alcohol is then added as long as a precipitate is formed. The mother liquor from the precipitate contains purified sphingomyelin. It is concentrated under diminished pressure and transferred into acetone. The purity of the material obtained in these stages depends largely on the nature of ligroin employed for solution. However, the poorest material obtained in this phase had the composition of Thudichum's sphingomyelin.

6. The final purification is accomplished by recrystallization of the substance from a solution containing equal parts of pyridine (Kahlbaum's) and chloroform.

Composition of sphingomyelin. Table I contains the results of analysis of a great number of samples of sphingomyelin. For economy of space only the final values are given. Sample 1 represents sphingomyelin which gave a negative orcin test. All the other samples gave a positive test, though of varying intensity. In some the color test was very faint.

The carbon and hydrogen estimations were made by the Drenstedt method; nitrogen by the Kjeldahl process, and phosphorus by the fusion method.

The *methyl estimation* was carried out according to the directions of Herzig and Meyer. The only modification in the process consisted in the use of two individual Rose metal baths for heating the

bulbs, instead of the sand bath employed originally. The temperature of a sand bath differs too much in different layers to permit of reliable information regarding the temperature of that part of the apparatus where the reaction takes place. The purity of reagents was tested.

TABLE II.

NUMBER OF SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF AGI	PER CENT NITROGEN	CH ₃	
				Calculated	Found
	grams	grams		per cent	per cent
4	0.2724	0.1954	3.36	5.40	4.57
5	0.2200	0.1638	3.29	5.27	4.75
6	0.3375	0.2456	3.29	5.27	4.64
7	0.2706	0.2542	3.32	5.33	5.99

Isolation and identification of choline. 35.0 grams of sphingomyelin were taken up with an equal weight of recrystallized barium hydrate in 200 cc. of water and heated in an autoclave at 120° for six hours. The reaction product was filtered. The filtrate was freed from barium and concentrated. The residue was extracted with absolute alcohol, the alcoholic extract filtered and concentrated. The residue was extracted with alcohol, again concentrated, and the final residue acidulated with hydrochloric acid and treated with chloroplatinic acid. The precipitate was recrystallized out of 60 per cent alcohol.

0.1196 gram of substance gave 0.0494 gram H₂O, 0.0846 gram CO₂ and 0.0382 gram Pt.

	Calculated for (C ₂₁ H ₄₄ (NOCl) ₂ PtCl ₆	Found:
C.....	19.48	19.29
H.....	4.58	4.13
Pt.....	31.65	32.00

Hydrolysis of sphingomyelin. Only the process as finally adopted will be given here. Many other conditions have been tested out and employed in course of the work, but all were found less satisfactory for one reason or another.

A given weight of the substance, an equal weight of barium hydrate crystals and ten times its weight of water were placed in an autoclave provided with a stirring arrangement, and heated

for forty-eight hours at 120°C. The reaction product was filtered. The filtrate contained choline, the residue the acids, the bases, and some intermediate products of hydrolysis. The entire residue was then taken up in a solution of 4 parts of 10 per cent hydrochloric acid and 1 part of 95 per cent alcohol, transferred to a hydrolysis flask provided with return condenser and mechanical stirrer and heated over an open flame for forty-eight hours. At the end of that time a layer of oil formed on the surface of the liquid. On cooling it formed a solid cake. It was found that the cake consisted of fatty acids, some esters, and the bases that were not choline. In order to saponify the esters the cake was dissolved in methyl alcohol, an excess of barium hydrate in methyl alcohol added, and the mixture heated (in a hydrolysis flask provided with a return condenser) for six hours in a water bath.

Separation of the acids from the bases and their purification. The acids are obtained by treating the reaction mixture of the previous experiment with acetone. A precipitate is formed which consists principally of fatty acids. For purification the barium salts are decomposed with hydrochloric acid and the fatty acids extracted with ether. The mixed acids obtained in this manner have as a rule the following composition: C = 76.2–76.8; H = 12.6–12.8. The isolation of lignoceric acid is accomplished in the following way. The mixed acids are extracted with petroleic ether boiling below 40°C. The extract consists principally of lignoceric acid. In order to purify the acid, the ethereal extract is freed from ether and the residue esterified by boiling with ethyl alcohol to which sulphuric acid was added to make a 5 per cent solution. On cooling to about 15° the ester separates in bright scales. These are recrystallized once out of slightly acidulated alcohol and once out of acetone. The substance melts at 56° and has the following composition:

0.1170 gram of substance gave 0.1342 gram H₂O and 0.3372 gram CO₂ (Dennstedt).

	Calculated for C ₂₄ H ₄₆ O ₂ .C ₂ H ₅ :	Found:
C.....	78.79	78.59
H.....	13.13	12.83

The cerebronic acid was separated from another experiment. The product obtained after hydrolysis with 4 parts of dilute hydro-

chloric acid and 1 part of alcohol was dissolved in methyl alcohol. This solution contained the bases, and the fatty acids and their esters. The free acid is (largely) cerebronic acid. Lignoceric acid is present principally in form of its ester. Hence adding to a methyl alcoholic solution of the products of hydrolysis a solution of barium hydrate in methyl alcohol one obtains the barium salt of cerebronic acid. The adhering lignoceric acid ethyl ester is then removed by means of ether. The barium salt of cerebronic acid is then decomposed by means of hydrochloric acid and the acid extracted with ether. If the resulting product still contains an admixture of lignoceric acid the latter can be removed as its lithium salt. In the experiment here reported this was not necessary. The acid obtained in this manner had the composition of cerebronic acid.

0.1088 gram of substance gave on combustion (Dennstedt) 0.1210 gram H_2O and 0.3022 gram CO_2 .

	Calculated for $C_{25}H_{40}O_8$	Found:
C.....	75.33	75.72
H.....	12.50	12.44

For the molecular weight estimation the substance was converted into its lead salt, which was washed first with water and then with acetone. The lead salt was finally decomposed in the usual way and the resulting acid employed for molecular weight estimation.

1.0266 grams of substance were dissolved in benzene and methyl alcohol and titrated first with $\frac{N}{2}$ and towards the end with $\frac{N}{10}$ alkali. It consumed the equivalent of 25.5 cc. of $\frac{N}{10}$ alkali.

	Calculated for $C_{25}H_{40}O_8$	Found:
Mol. Wt.....	398	402

The separation of the bases. A great many experiments were made with a view of obtaining the principal base in a pure condition. Only the final form of analysis will be given here. The crude bases are obtained in the following way. The filtrate obtained after filtering off the barium salts of the fatty acids is evaporated to dryness under diminished pressure. The residue is redissolved in acetone. The soaps that were not removed by the first acetone treatment remain insoluble on the second extraction. The insoluble part is removed by filtration, and the filtrate is concentrated to dryness. If necessary the acetone treatment may be repeated. The final residue containing the bases and some esters of the fatty

acids is dissolved in very little alcohol. From this solution the bases can be isolated in form of their sulphates by adding an alcoholic solution of sulphuric acid until the reaction of the mixture turns acid to litmus. The sulphates obtained in this manner contained about 63 per cent of C and about 10.5 per cent of H. Hence it was concluded to saturate the alcoholic solution of the bases with hydrogen in the presence of palladium. It was found that the fractionation was accomplished easier on the dihydro bases.

The solution of the saturated bases was then treated with alcoholic sulphuric acid and the sulphates of the bases freed from adhering esters by means of dry ether. These mixed sulphates were repeatedly fractionated out of a solution containing equal parts of alcohol and of chloroform. By this treatment on several occasions it was possible to obtain a fraction which in its composition approached that of $(C_{17}H_{37}NO)_2 H_2SO_4$ and another fraction which contained only about 62 per cent of carbon and sometimes fractions that analyzed well for sphingosine sulphate.

However, the many purifications consumed so much material that as yet we are not in possession of a quantity sufficient to make derivatives of the bases. It is hoped that this end will be achieved in the future.

Results of analysis of several of the higher fractions.

Sample No. 565. 0.1174 gram of substance gave on combustion 0.1213 gram H_2O and 0.2770 gram CO_2 .

0.1000 gram of substance employed for Kjeldahl nitrogen estimation, required 3 cc. of $\frac{N}{10} H_2SO_4$ for neutralization.

Sample No. 657. 0.1182 gram of substance gave 0.1200 gram H_2O and 0.2781 gram CO_2 .

0.1495 gram of substance used for Kjeldahl nitrogen estimation, required for neutralization 4.95 cc. $\frac{N}{10} H_2SO_4$.

Sample No. 768. 0.1157 gram of substance gave 0.1202 gram H_2O and 0.2690 gram CO_2 .

0.1976 gram of substance used for Kjeldahl nitrogen estimation, required 5.85 cc. $\frac{N}{10} H_2SO_4$ for neutralization.

0.2964 gram of substance gave 0.1066 gram $BaSO_4$.

	Calculated for $(C_{17}H_{37}NO)_2 H_2SO_4$:	No. 565	Found: No. 657	No. 768
C.....	63.80	64.34	64.17	63.40
H.....	11.85	11.56	11.38	11.63
N.....	4.37	4.20	4.65	4.15
S.....	5.00			4.94

The sample No. 76S melted with effervescence at 280° (corr.) and had the following rotation:

0.1000 gram of substance in 2.0 cc. of alcohol containing sulphuric acid (total weight, 1.8305) rotated in 1 dm. tube -0.70 at $t = 25^{\circ}\text{C}$.

$$[\alpha]_D^{25} = -12.8^{\circ}$$

The number of substances that gave lower values for carbon and hydrogen was very great. There were analyzed at least about thirty samples. However, little significance can be attached to these values, hence they are not reported here.

PURIFICATION AND MELTING POINTS OF SATURATED ALIPHATIC ACIDS.

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In course of our work on lipoids we frequently were in need to refer to the melting points of the known higher fatty acids. On several occasions we had sufficient ground to doubt the correctness of the accepted melting points, and hence we were led to reinvestigate the aliphatic acids beginning with $C_{11}H_{22}O_2$ and ending with $C_{16}H_{32}O_2$ in regard to their physical constants.

The melting points found by us were generally higher than those given in the literature. It was also found that the purest acids after distillation even under very low pressure as a rule do not show their maximum melting point. In order to obtain the maximum several recrystallizations out of acetone were required. An irregularity was observed in connection with the melting point of lauric acid. This acid is considered to have the structure of the normal dodecylic acid. As a rule the melting point of the normal acids with an even number of carbon atoms in the chain is higher than of the following acid with an uneven number of carbon atoms. Thus, $C_{14}H_{28}O_2$ = M.P., $58^{\circ}C$, and $C_{13}H_{26}O_2$ = M.P., $54^{\circ}C$, whereas the melting point of lauric acid, $C_{12}H_{24}O_2$ is $48^{\circ}C$, and that of tridecylic, $C_{13}H_{26}O_2$ is $51^{\circ}C$. We plan to investigate into the causes of this abnormality.

EXPERIMENTAL PART.

The fatty acids used in the following work were prepared by the method recently described by us¹ or were purchased from Kahlbaum or Schuchardt. In each case at least three different lots of

¹ Levene and West: *This Journal*, xvi, p. 475, 1914.

the acid were purified using the following methods: (1) The acid was recrystallized from acetone two or three times, distilled in vacuum, and then recrystallized from acetone. (2) The acid was purified through the lead salt (the lead salt in most cases being extracted with acetone to remove any ester present), and then recrystallized several times from dry acetone. (3) The crude acid was distilled in vacuum (Geryke pump) and then recrystallized from acetone until the melting point was constant. We have purposely avoided the use of alcohol as there is always more or less esterification of the acid, even if the acid is boiled with the alcohol for a very short time.² The presence of a very small amount of ester naturally would lower the melting point of the acid. It has been our experience that the melting points of the acids are low after the distillation in vacuum. While Holland recommends the distillation of the acid or its ester as a method of purification and says that crystallization is a finishing rather than an initial process of purification, we have found that crystallization is a very important process in cases where the correct melting point is desired. It is true that, in the cases of the lower members of the series, the process is rather wasteful of material, though, of course, all the crude material can be recovered from the mother liquor.

Undecylic acid, $C_{11}H_{22}O_2$. Undecylic acid was obtained by reducing undecylenic acid with hydrogen and colloidal palladium. Six cc. of Schuchardt's acid were dissolved in about 25 cc. absolute alcohol, the solution warmed to 60–70° and shaken with 0.1 gram colloidal palladium, dissolved in a little water, in an atmosphere of hydrogen. When no more hydrogen was absorbed, the reaction product was filtered from the palladium (a few drops of acetic acid may be necessary to coagulate the palladium), warmed on the water bath and treated with a slight excess of methyl alcoholic lead acetate. The lead salt is decomposed as usual with hydrogen sulphide in toluene. The acid boiled at 164° under 15 mm. pressure and melted at 28–29°. Recrystallized from dry acetone at –10° it melts at 29–30°. Because of its great solubility it is not practical to purify it further. Krafft³ gives 28.5 as the melting point.

² Holland: *Journ. of Ind. and Eng. Chem.*, iii, pp. 171–3, 1911. This does not hold true for the higher fatty acids (above C_{20}).

³ *Ber. d. deutsch. chem. Gesellsch.*, xi, p. 2219, 1878; xii, p. 1668, 1879.

0.1200 gram of substance gave 0.3115 gram CO_2 and 0.1262 gram H_2O .

0.9800 gram of the acid, dissolved in absolute methyl alcohol and benzene, required 53 cc. $\frac{N}{10}$ NaOH, using phenolphthalein as an indicator.

	Calculated for $\text{C}_{11}\text{H}_{22}\text{O}_2$:	Found:
C.....	70.9	70.80
H.....	11.8	11.77
Mol. Wt.....	186.0	185.00

The acid was characterized by changing it into the *amide*,⁴ which, recrystallized from absolute alcohol, melted at 103° .

This method of reducing unsaturated fatty acids has also been successfully used in the preparation of behenic acid from erucic acid. It is as convenient as the palladium black method, which is recommended by Parnas,⁵ or the use of finely divided nickel heated to 170° , as used by Pickard and Kenyon.⁶

Lauric acid, $\text{C}_{12}\text{H}_{24}\text{O}_2$. Kahlbaum's acid melts at $46-48^\circ$, and boils at $141-142^\circ$ under a pressure of 0.6–0.7 mm. Twice recrystallized out of acetone it melts at $47.5-48^\circ$. The other methods of purification gave a similar melting point. Krafft⁷ gives a melting point of 43.5° .

Tridecylic acid, $\text{C}_{13}\text{H}_{26}\text{O}_2$. Tridecylic acid was prepared by the oxidation of α -hydroxy-myristic acid with potassium permanganate in acetone solution. In this case the potassium salt of the acid is soluble in acetone and most of the desired product is found in the filtrate from the manganese dioxide. The acid boils at $202-203^\circ$ under 17 mm. pressure, and melts at $47-48^\circ$. Twice recrystallized from acetone it melts at $50-51^\circ$. The melting points given in the literature are, Krafft,⁸ 40.5° ; Le Sueur,⁹ 42.5° .

0.557 gram of substance, dissolved in a mixture of equal parts of benzene and methyl alcohol, required 26 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $\text{C}_{13}\text{H}_{26}\text{O}_2$:	Found:
Mol. Wt.....	214.0	214.3

⁴ Ehestädt: Dissertation, Freiburg, 1886.

⁵ *Biochem. Zeitschr.*, xxii, p. 428, 1909.

⁶ *Journ. Chem. Soc.*, ciii, p. 1947, 1913.

⁷ *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1666, 1879; xiii, p. 1415, 1880; Hintz: *Ann. d. Chem.*, xcii, p. 294, 1854.

⁸ *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1670, 1879.

⁹ *Journ. Chem. Soc.*, lxxxvii, p. 1898, 1905.

The acid was characterized as the amide, which, recrystallized from absolute alcohol, melts at 98.5° E. Lutz¹⁰ gives the same melting point for the product obtained from myristic acid amide.

Myristic acid, $C_{14}H_{28}O_2$. The sample of Schuchardt's acid used melted at $53.5\text{--}54^{\circ}$ and boiled at $185\text{--}187^{\circ}$ at 0.8–0.9 mm. pressure. The distilled product melted at $56\text{--}57^{\circ}$. This melting point was raised to $57.5\text{--}58^{\circ}$ after three recrystallizations. The melting point given in the literature is $53.5\text{--}54^{\circ}$.¹¹

Pentadecylic acid, $C_{15}H_{30}O_2$. Pentadecylic acid was obtained by the oxidation of α -hydroxy-palmitic acid.

Methyl α -hydroxy-palmitate, prepared in the usual way by boiling the acid with absolute methyl alcohol containing about 10 per cent sulphuric acid, forms colorless crystals from absolute methyl alcohol, then from acetone, melting at $59\text{--}60^{\circ}$.

0.1200 gram of substance gave 0.3128 gram CO_2 and 0.1276 gram H_2O .

	Calculated for $C_{17}H_{34}O_2$	Found:
C.....	71.25	71.09
H.....	11.97	11.90

Ethyl α -hydroxy-palmitate was prepared as given above. Recrystallized from absolute alcohol it melts at $55.5\text{--}56.5^{\circ}$.

0.1200 gram of substance gave 0.3161 gram CO_2 and 0.1287 gram H_2O .

	Calculated for $C_{19}H_{38}O_2$	Found:
C.....	71.93	71.84
H.....	12.08	12.00

Oxidized in the usual way, and purified by distillation and crystallization from acetone, a product was obtained which melted at 53° . This was fractionally precipitated with lead acetate and the three products obtained fractionally crystallized from dry acetone. No change in the melting point was observed.

The acid was then prepared according to the directions given for oxidation of sphingosine:¹² 10 grams of the hydroxy acid were dissolved in 100 cc. of glacial acetic acid and a solution of 5.5 grams

¹⁰ Ber. d. deutsch. chem. Gesellsch., xix, p. 1439, 1886.

¹¹ Krafft: Ber. d. deutsch. chem. Gesellsch., xii, p. 1669, 1879; xiii, p. 1415, 1880; Noerdlinger: Ibid., xix, p. 1893, 1886; Heintz: Ann. d. Chem., xcii, p. 292, 1854.

¹² This Journal, xvi, p. 549, 1914.

of chromic acid in acetic acid slowly added from a dropping funnel, the acetic acid removed with steam and the chromium containing product distilled in vacuum. This acid, when redistilled and twice recrystallized from acetone, again melted at 53–54°.

0.1200 gram of substance gave 0.3284 gram CO_2 and 0.1319 gram H_2O .

1.000 gram of the acid required 41.3 cc. $\frac{N}{10}$ NaOH for neutralization.

Calculated for
 $\text{C}_{15}\text{H}_{30}\text{O}_2$:

	Calculated for $\text{C}_{15}\text{H}_{30}\text{O}_2$:	Found:
C.....	74.4	74.3
H.....	12.4	12.3
Mol. Wt.....	242.0	242.1

The melting points given for pentadecylic acid are: Krafft,¹³ 51°; LeSueur,¹⁴ who prepared it by oxidizing the aldehyde, 53°; and Majima and Nakamura,¹⁵ who prepared it by hydrolysis of the cyanide, 52°.

The amide obtained from this acid melted at 102–103°, which is that given in the literature.

Palmitic acid, $\text{C}_{16}\text{H}_{32}\text{O}_2$. Palmitic acid, as above, showed a melting point of 63.5–64°.

The following table gives a comparison of our melting points with those previously recorded in the literature:

ACID	MEYER-JACOB- SON	LE SUEUR	LEVENE AND WEST
$\text{C}_{11}\text{H}_{22}\text{O}_2$	28.0°		29.3°
$\text{C}_{12}\text{H}_{24}\text{O}_2$	44.0°		48.0°
$\text{C}_{13}\text{H}_{26}\text{O}_2$	40.5°	42.5°	51.0°
$\text{C}_{14}\text{H}_{28}\text{O}_2$	54.0°	53.5–54°	58.0°
$\text{C}_{15}\text{H}_{30}\text{O}_2$	51.0°	53.0°	54.0°
$\text{C}_{16}\text{H}_{32}\text{O}_2$	62.6°	62.5°	63–64°

¹³ *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1671, 1879.

¹⁴ *Journ. Chem. Soc.*, ciii, p. 1898, 1905.

¹⁵ *Ber. d. deutsch. chem. Gesellsch.*, xlvi, p. 4089, 1913.

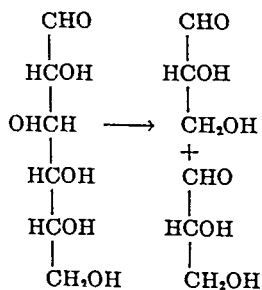
ON THE ACTION OF TISSUES ON METHYL GLUCOSIDES, TETRAMETHYL GLUCOSIDES AND NATURAL DISACCHARIDES.

By P. A. LEVENE AND G. M. MEYER.

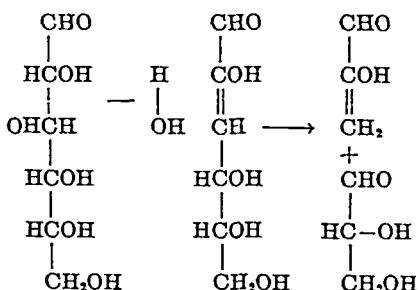
(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

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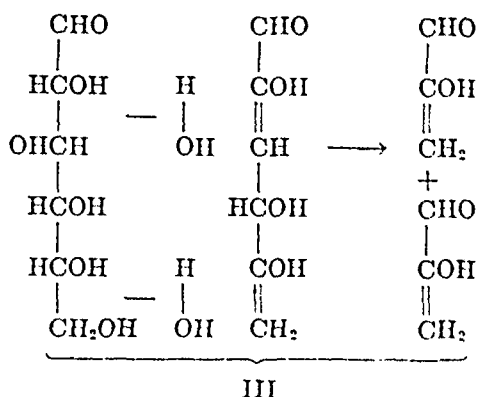
The volume of work done on the mechanism of sugar combustion in the animal organism is very considerable. The information thus far furnished by it is insignificant. It is known that in the process of sugar oxidation lactic acid formation does take place, probably through the intermediate formation of methyl glyoxal. The steps that lead up to methyl glyoxal are not known. The glucose molecule may be dissociated into two molecules of glyceric aldehyde. It may also undergo such a transformation which will lead to its further dissociation into methyl glyoxal and into glyceric aldehyde. The glucose molecule may also suffer a change leading to its dissociation into two molecules of methyl glyoxal. The three possibilities may be graphically expressed in the following manner:



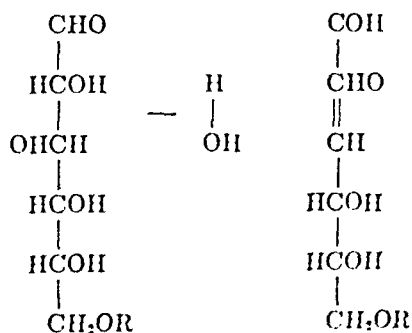
I



II



A priori all these reactions are possible. In reality there is no experimental evidence either for or against any one of the possibilities. From the graphic expressions of the reactions it is made obvious that the hypothetic intermediate body is formed through a loss of one or more molecules of water by the original hexose molecule. This reaction presumes a certain degree of mobility of the various atoms and hydroxyl groups in the sugar molecule. On the basis of this one conceives the activity of sugar-splitting enzymes as playing on the mobility of the various hydrogen atoms or hydroxyl radicals of the sugar molecule. Naturally one should expect that by stabilization of one or more hydroxyl groups the resistance of the hexose molecules against the action of the enzyme will be enhanced. For instance, one may conceive that by stabilizing the hydroxyl in ϵ -position the first hydrogen and hydroxyls in position α and β will function normally and hence the altered molecule may still yield half of the normal quantity of methyl glyoxal and of lactic acid. This is made obvious by the following graphic expression:



in the experiments of Voit is due to the same factor as in our experiments.

The results of our experiments seem to be of special interest at the present moment when several authors have advanced the view that glucose prior to its combustion by the animal organism enters into some rather mysterious chemical union with other substances. It is hard to reconcile the results of our experiments with such speculations.

EXPERIMENTAL PART.

Tissues. Rabbit kidneys were removed aseptically from exsanguinated animals, finely cut and immediately added to the sugar solutions.

Sugar solutions. All disaccharide solutions were of 5 per cent in 10 per cent Henderson phosphate solution and sterilized by filtering through sterile Berkefeld filters. Their sterility was tested before adding the tissues by means of cultures and smears. The tetramethyl glucose was prepared according to the method of Purdie and Irvine. It was redistilled under diminished pressure and boiled constant at 122° at 0.5 mm., and melted at 81°. Glucose-phosphoric acid was prepared according to Neuberg. A PO_4 determination of the calcium salt showed that it contained 8.9 per cent P. Five and eight per cent solutions were used. For the other sugars, preparations of Kahlbaum were used. All solutions were allowed to stand at 37° for thirty-six hours. Samples of each were removed for analyses immediately after adding the kidney tissue. In the experiments with tetramethyl glucose and glucose-phosphoric acid the sugar solution was divided into equal parts, and to each was added one kidney. One portion was reserved as control and analyzed immediately. This procedure was followed to eliminate any possible contamination of the main portion while removing the control sample for analysis.

Methods of analysis. The reducing power of the sugar solutions was determined by Fehling solution and estimating the reduced copper according to Volhard. Determination of reducing power was also made on the sugar after hydrolysis with 2 per cent hydrochloric acid according to the standard methods for each particular sugar.

Bacteriological control. All solutions were tested after thirty-six hours' incubation by means of cultures and only those which were found sterile received further consideration.

The bacteriological examinations were made by Dr. Martha Wollstein to whom we desire to express our appreciation.

Maltose.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before.....	2	17.30	8.65	
After.....	2	21.80	10.90	+2.25
Hydrolyzed:				
Before.....	1	14.90	14.90	
After.....	1	13.00	13.00	-1.90
II. Before.....	2	14.00	7.00	
After.....	2	17.00	8.50	+1.50
Hydrolyzed:				
Before.....	1	14.50	14.50	
After.....	1	12.70	12.70	-1.80
III. Before.....	2	14.00	7.00	
After.....	2	16.80	8.40	+1.40
Hydrolyzed:				
Before.....	1	14.50	14.50	
After.....	1	11.50	11.50	-3.00

Lactose.

I. Before.....	2	18.00	9.00	
After.....	2	18.00	9.00	
Hydrolyzed:				
Before.....	1	12.30	12.30	
After.....	1	12.20	12.20	
II. Before.....	2	17.80	8.90	
After.....	2	17.70	8.85	
Hydrolyzed:				
Before.....	2	26.50	26.50	
After.....	2	26.70	26.50	
III. Before.....	2	18.40	9.20	
After.....	2	18.40	9.20	
Hydrolyzed:				
Before.....	1	14.00	14.00	
After.....	1	13.80	13.80	

Sucrose.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before.....	4	0.00	0.00	
After.....	4	0.00	0.00	
Hydrolyzed:				
Before.....	1	14.40	14.40	
After.....	1	14.40	14.40	
II. Before.....	4	0.00	0.00	
After.....	4	0.00	0.00	
Hydrolyzed:				
Before.....	1	14.80	14.80	
After.....	1	14.60	14.60	

 β -Methyl glucoside.

I. Before.....	2	0.00	0.00	
After.....	2	1.50	0.75	+0.75
Hydrolyzed:				
Before.....	1	13.30	13.30	
After.....	1	10.30	10.30	-3.00
II. Before.....	5	0.00	0.00	
After.....	5	1.20	0.24	+0.24
Hydrolyzed:				
Before.....	1	13.90	13.90	
After.....	1	13.00	13.00	-0.90

 α -Methyl glucose.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before.....	5	0.00	0.00	
After.....	5	0.00	0.00	
Hydrolyzed:				
Before.....	1	10.90	10.90	
After.....	1	10.70	10.70	
II. Before.....	5	0.00	0.00	
After.....	5	0.00	0.00	
Hydrolyzed:				
Before.....	1	11.40	11.40	
After.....	1	11.60	11.60	

Tetramethyl glucose.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before.....	5	14.00	2.80	
After.....	5	14.20	2.84	
II. Before.....	4	14.60	3.65	
After.....	4	14.80	3.70	
III. Before.....	5	8.60	1.72	
After.....	5	8.90	1.78	

Glucose-phosphoric acid.

I. Before.....	2	8.50	4.25	
After.....	2	8.30	4.15	

ON CEREBRONIC ACID.

FOURTH PAPER.¹

ON THE CONSTITUTION OF LIGNOCERIC ACID.

By P. A. LEVENE AND C. J. WEST.

(From the Laboratories of the Rockefeller Institute for Medical Research,
New York.)

(Received for publication, June 16, 1914.)

In a previous publication² the conclusion was reached that lignoceric acid had the structure of the normal tetracosanic acid. The view was based on the melting point of the tetracosane obtained on reduction of lignoceric acid. The melting point of the hydrocarbon obtained from lignoceric acid on reduction with hydriodic acid was 51°C and that given in the literature for the normal tetracosane was also 51°C. Hence the two substances were considered identical. Since then Hans Meyer³ has published the results of his work on lignoceric acid which led him to the belief that lignoceric acid was not identical but isomeric with the normal tetracosanic acid. This conclusion did not harmonize with the one based on the melting point of the hydrocarbon.

Hence there arose the suspicion that the melting point found by previous observers for the normal tetracosane was incorrect. This consideration led us to undertake a revision of the melting points of a number of the higher hydrocarbons. The work is carried out in coöperation with Mr. J. Van der Scheer. In this communication we report only the results obtained on the hydrocarbon obtained through the action of magnesium on dodecyl iodide and on the hydrocarbon obtained from lignoceric acid. This latter hydro-

¹ The preceding papers are to be found in this *Journal*, xii, p. 381, 1912; xiv, p. 257, 1913; xv, p. 193, 1913. In the table in paper III, the melting points of the two esters are interchanged.

² This *Journal*, xv, p. 193, 1913.

³ H. Meyer, L. Brod, and W. Soyka: *Monatsh. f. Chem.*, xxxiv, p. 1113, 1913.

carbon was obtained by reducing lignoceric ester to the corresponding alcohol; the alcohol was then converted into the iodide, and this reduced to the hydrocarbon.

It was found that the hydrocarbon prepared in this manner had the same melting point as found by us previously, namely, 51°C. On the other hand, the melting point of the normal hydrocarbon was found at 55°C. On the basis of this, our original view regarding the structure of lignoceric acid has to be abandoned, and lignoceric acid will have to be regarded as isomeric and not identical with the normal tetracosanic acid. Furthermore, the original view on the structure of cerebronic acid will have to be modified, as in this acid also the carbon atoms are united in a branched and not in a normal chain.

EXPERIMENTAL PART.

Dodecyl iodide. Ten grams of dodecyl alcohol, 1.4 grams of red phosphorus and 7 grams of iodine were heated in a metal bath at 170° for one hour. The reaction product was taken up in ether, the solution washed with water, the iodine removed with sodium thio-sulphate and the solution dried with anhydrous sodium sulphate. After removing the ether, the iodide boiled at 145–150° under 0.7 mm. pressure.

I. 0.1790 gram of substance gave 0.1418 gram AgI (Carius).

II. 0.1820 gram of substance gave 0.1432 gram AgI (Carius).

	Calculated for $C_{12}H_{25}I$	I Found:	II
I.....	42.79	42.82	42.53

A preparation in which 10 grams of the alcohol, 40 cc. glacial acetic acid and 120 grams of hydroiodic acid (d. 1.7) were boiled for six hours, contained only 37.70 per cent iodine, showing that part of the iodide had been reduced to the corresponding hydrocarbon. It was not possible to separate these by fractional distillation.

Normal tetracosane. This hydrocarbon was prepared by the action of magnesium upon the above iodide in ordinary dry ether.⁴ The ether need not be as carefully dried as for the usual Grignard

⁴ This is a general reaction for aliphatic iodides above hexyl iodide, cf. Wren, *Organometallic Compounds of Zinc and Magnesium*, p. 14.

reaction. 0.9 gram of magnesium and about 100 cc. ether are placed in a double-necked flask, fitted with a condenser and a stirrer, and 9 grams of dodecyl iodide, dissolved in ether, gradually added. The reaction is warmed on the water bath until all the magnesium has gone into solution. The reaction product was allowed to stand over night. On cooling there appeared in the ether a deposit of brilliantly shining scales (tetracosane). The mixture was then transferred into acidulated water, and washed in a separatory funnel until all magnesium was removed. By this treatment the Grignard magnesium complex is decomposed. The ethereal solution was warmed and dried with anhydrous sodium sulphate, and then allowed to stand over night in the refrigerator. Tetracosane appeared in the form of bright shining scales. All the dodecane remained in solution. Tetracosane was then purified by fractional distillation. It boiled at 237–240°C under 15 mm. pressure. It was again recrystallized out of ether. The substance melted at 55°C. The melting point given in literature is 51.1°C.⁵

0.1123 gram of substance gave 0.3501 gram CO₂ and 0.1484 gram H₂O.

	Calculated for C ₂₄ H ₅₀	Found:
C.....	85.10	85.02
H.....	14.90	14.79

Iso-tetracosyl alcohol. Thirty grams of ethyl lignocerate were reduced with 30 grams sodium and amyl alcohol according to Bouveault and Blanc.⁶ The alcohol was extracted from the soap with ether, the solution dried and after the removal of the ether, distilled in vacuum. It boils at 220° under 0.8 mm. pressure. Recrystallized from ligroin or chloroform, it melts at 72°. The yield is about 4 grams. Since nearly all the acid is recovered, the method is not so wasteful of material as the above yield would indicate.

0.1200 gram of substance gave 0.3571 gram CO₂ and 0.1218 gram H₂O.

	Calculated for C ₂₄ H ₅₀ OH	Found:
C.....	81.26	81.16
H.....	14.22	14.30

⁵ Kraft: *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1720, 1882; Mabery: *Amer. Chem. Journ.*, xxviii, p. 165, 1902.

⁶ *Compt. rend. Acad. d. sci.*, cxxxvi, p. 1676, 1903; cxxxvii, pp. 60 and 323, 1903.

Iso-tetracosyl iodide. This was prepared as above, using 9 grams of the alcohol, 3.5 grams of iodine and 1 gram of red phosphorus. Recrystallized from ether, the iodide melts at 48°.

0.1704 gram of substance gave 0.0870 gram AgI (Carius).

	Calculated for $C_{24}H_{50}I$:	Found:
I.....	27.34	27.60

Iso-tetracosane. The hydrocarbon corresponding to lignoceric acid has already been described by us as melting at 51°. At that time it was prepared by the action of concentrated hydroiodic acid upon the acid. We have now prepared it by the action of zinc upon the iodide in glacial acetic acid containing hydrochloric acid. The iodide and zinc are suspended in the acetic acid, and hydrochloric acid gas passed in so that there is a constant evolution of hydrogen. At the end of the reduction, the acid is distilled off in vacuum, the hydrocarbon taken up in ether and washed with water. The hydrocarbon was purified by distillation at 9 mm. pressure and 222–225°C. The distillate was recrystallized out of ether. Isotetracosane separated out in shining plates and has a melting point of 51–51.5°C.

0.1133 gram of substance gave 0.3525 gram CO_2 and 0.1488 gram H_2O .

	Calculated for $C_{24}H_{50}$:	Found:
C.....	85.10	84.84
H.....	14.90	14.70

ON SPHINGOSINE.

THIRD PAPER.¹

THE OXIDATION OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

BY P. A. LEVENE AND C. J. WEST.

(From the Laboratories of the Rockefeller Institute for Medical Research,
New York.)

(Received for publication, June 16, 1914.)

In the previous work on the oxidation of sphingosine and dihydrosphingosine evidence was furnished that tridecylic acid was formed from the first substance and under the same conditions pentadecylic acid was obtained from the second. On the basis of this the structure of sphingosine was expressed in the following manner:



The character of the carbon chain and the configuration of the substituting groups was not established. The failure to determine the nature of the carbon chain was due to the fact that the melting point found by us for the tridecylic acid obtained on oxidation of sphingosine was higher than the one at that time accepted for the normal tridecylic acid and the same applied to the pentadecylic acid obtained on oxidation of dihydrosphingosine.

Hence we were led to revise the older data on the melting points of the aliphatic acids from $\text{C}_{11}\text{H}_{22}\text{O}_2$ to $\text{C}_{18}\text{H}_{36}\text{O}_2$. The results are reported in a separate communication. On the other hand, we prepared the acids from sphingosine and its dihydro derivative in larger quantities in order to permit a higher degree of purification.

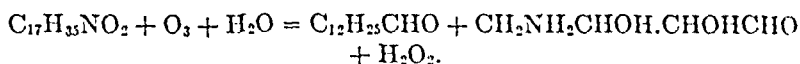
As a result of this work it was found on one hand that the generally accepted melting point for tridecylic acid was much below the correct one, and the melting point of pentadecylic acid reported by us in the previous paper was higher than the correct one.

The corrected melting point of the acids obtained from sphingosine and from its dihydroderivative, and also the melting points

¹ This *Journal*, xi, p. 547, 1912; xvi, p. 549, 1914.

of their amides harmonize exactly with the corrected melting points of the corresponding normal acids and of their amides respectively. Hence, it becomes evident that the carbon atoms in the molecule of sphingosine are united in a normal chain.

An attempt was made to solve the configuration of the substituting groups by the action of ozone on sphingosine. It was expected, on the basis of the experience of Harries, to obtain by cleavage of the ozonide tridecylic aldehyde and an amino-tetrose according to the following reaction:



For convenience of identification it was concluded to convert one into tridecylic acid and the other into tartaric acid. The tridecylic acid was isolated without great difficulty. The tartaric acid could not be isolated with certainty. In one experiment there formed a small quantity of crystals, which had the appearance of the calcium salt of mesotartaric acid. However, the quantity was insufficient for analysis, hence the conclusion regarding the configuration of the substituting groups will have to be deferred until future date.

EXPERIMENTAL PART.

The fatty acid obtained from the oxidation of sphingosine was again recrystallized from acetone, when it melted at 48–49°. Mixed with a sample of carefully purified tridecylic acid, it showed no depression of the melting point.

The acid was then changed into the amide by the usual method. Twice recrystallized from absolute alcohol it melted at 98–99°. The mother liquors gave a product, which recrystallized from 85 per cent alcohol, melted at 96°. This showed that the product was homogeneous. When mixed with the amide of normal tridecylic acid, the melting point was 97–98°. This leaves no doubt that the acid obtained by the oxidation of sphingosine is normal tridecylic acid.

Oxidation of dihydrosphingosine.

About 30 grams of dihydrosphingosine were oxidized in 5-gram lots according to the directions given in the first paper. The green product obtained from the steam distillation was dried in ether

solution, the ether removed and the product distilled in vacuum. We have tried to remove the chromium by heating with strong potassium hydroxide solution, but unsuccessfully. The colorless product obtained upon distillation was twice recrystallized from acetone. It melted at 53°.

0.5000 gram of substance required 20.0 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{15}H_{31}O_2$:	Found:
Mol. Wt.....	242	250

The acid was then changed into the acid amide. This product, recrystallized from absolute alcohol, melted at 102° and when mixed with a sample of amide from normal pentadecylic acid, melted at 102–103°.

This establishes the constitution of the pentadecylic acid as the normal acid.

Action of ozone upon sphingosine.

Five grams of sphingosine sulphate were dissolved in 50 cc. of chloroform and the solution treated with ozone for a period varying from one to two hours. The preparation we used gave a red color in chloroform, more or less pronounced, and we found that when this color disappeared, that is when the solution became water-white, the reaction might be considered as ended. Under these conditions about one-eighth of the nitrogen was split off. If the ozone stream was continued longer, more and more of the amino nitrogen was destroyed.

The chloroform was distilled off the ozonide and the residue decomposed with water. It may be boiled for about ten minutes or shaken over night at room temperature.

The product was then extracted with ether. This removed the longer portion of the carbon chain. After removing the ether the product was taken up in acetone and oxidized with potassium permanganate. The acetone was filtered off, and the filtrate and precipitate worked up for fatty acid. The crude fatty acid was dissolved in methyl alcohol and neutralized to phenolphthalein with methyl alcoholic barium hydroxide. The precipitate was filtered off, decomposed with dilute hydrochloric acid, and reprecipitated several times. In some cases amyl alcohol was used to prevent

foaming during the time of ozonizing. The valeric acid which resulted from the oxidation of this alcohol was washed out with hot water, and the acid finally purified through the lead salt. Recrystallized from acetone, the acid melted at 48–49°. One lot was characterized by changing it into the amide, which melted at 98–99°. This shows the acid to be normal tridecyclic acid, as would be expected from the results of the first oxidation experiment.

0.5000 gram of substance required 23.2 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{13}H_{25}O_2$:	Found:
Mol. Wt.....	214	215.5

The aqueous portion of the ozonide, after decomposition and extraction with ether, contained amino nitrogen, and reduced Fehling's solution. Theoretically one should expect in that solution the presence of an amino-tetrose. The isolation of this, or of a derivative of it, proved of great difficulty. Hence it was concluded to oxidize the aqueous part of the reaction product with nitric acid in hope to obtain tartaric acid. The aqueous solution, to which an equal part of strong nitric acid (sp. gr. 1.5) was added, was kept over night at 40°C, and the reaction product then rapidly evaporated on the water bath. In order to enhance evaporation, the operation was carried out in large watch glasses and in very small portions. The residues were taken up in water, boiled with calcium carbonate, allowed to cool and filtered. The filtrates on addition of ammonia water formed a precipitate. This was soluble in dilute hydrochloric acid and could be reprecipitated by ammonia. Once the precipitate formed in this manner had the calcium content of calcium tartarate.

0.1000 gram of substance, dried in xylol bath over phosphorous pentoxide and under diminished pressure, gave 0.0298 gram CaO.

	Calculated for $C_{13}H_{25}O_2Ca$:	Found:
CaO.....	29.7	29.8

It was then attempted to purify another sample prepared in the same manner following the directions of Anschütz.² On long standing there appeared a crystalline sediment. The crystals had the typical appearance of the calcium salt of mesotartaric acid, but the quantity was too small to permit an analysis.

² *Ann. d. Chem.*, ccxxvi, p. 191, 1884.

THE PARENTERAL UTILIZATION OF DISACCHARIDE SUGARS.¹

BY ALBERT G. HOGAN.

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(Received for publication, June 22, 1914.)

The earlier literature on the behavior of carbohydrates introduced parenterally in the organism has been summarized by Albertoni (1914), Mendel and Mitchell (1905), Mendel and Kleiner (1912), and others. The trend of the results, which need not be repeated here, has been such as to indicate an apparent inability on the part of the animal organisms to metabolize more complex carbohydrates, such as disaccharides and polysaccharides, except in cases where some device—for example, a carbohydrate-digesting enzyme—was discovered to be present in the blood or tissues. In the latter event the conversion of the product introduced parenterally could be carried to the stage from which it is normally metabolized in the body. The consensus of evidence, derived from the extent to which carbohydrates are easily utilized when introduced by the oral path, but reappear in the urine when they are injected subcutaneously, intravenously or intraperitoneally, has been that sucrose and lactose, in particular, among common sugars cannot be readily utilized parenterally. The disappearance of maltose is easily accounted for by the well known presence of maltase in the blood.

The recovery of sucrose or lactose in the urine after parenteral introduction of these sugars has frequently not been quantitatively complete. Analytical difficulties will undoubtedly explain part of the inconsistencies hitherto reported in this field. Recently the work of Abderhalden and his collaborators on the appearance of appropriate enzymes in the blood serum after in-

¹ The experimental data are taken from the dissertation presented by the author for the degree of Doctor of Philosophy, Yale University, 1914.

roduction of substances normally foreign to the circulation has called attention to a different aspect of the matter. The problem of developing enzymes in the blood stream under the conditions here referred to is by no means as new as it may seem to many. Earlier attempts with sugars in this laboratory and elsewhere have commonly resulted in a negative outcome. Abderhalden however states that under suitable experimental conditions the blood may exhibit enzymes that will hydrolyze lactose and sucrose. His earlier results (1910a) were inconstant, though a considerable number was positive. Later (1910b) the procedure was modified somewhat, and Abderhalden stated that parenteral injection of sucrose, according to his methods, was always followed by the appearance of sucrase or lactase in the serum. His newer procedure was to make injections of small quantities of sugar. He then observed that a single injection of lactose or sucrose was sufficient to induce the formation of the enzyme. When subcutaneous injections were made, he used only 10 cc. of a 5 per cent, or at most a 10 per cent solution, and the enzyme appeared in seven or eight hours. When intravenous injections were made, such small quantities as 2 cc. of a 5 per cent solution were often used, and the enzyme appeared in fifteen minutes. The serum enzymes obtained after injecting either lactose or sucrose would hydrolyze both of those sugars. Abderhalden's method of detecting these enzymes was to place highly diluted serum from the experimental animals, along with the carbohydrates under observation, in small polariscope tubes, and determine the optical rotation at various intervals. Obviously the variations in rotation were very small. In some cases the optical activity was checked by the copper reduction method.

The findings of Abderhalden were, in a way, confirmed by Heilner (1911), using a different method. He injected approximately 30 grams of sucrose into rabbits subcutaneously, and estimated the amount excreted in the urine. In some cases, large amounts, varying from 1.1 to 6.6 grams, could not be recovered. Heilner believed that sucrase had been developed in the rabbits. He also estimated the respiratory exchange and observed an increased elimination of carbon dioxide after administering the sucrose. This, he believed, was due to oxidation of the sucrose introduced.

Lombroso (1912) attempted to confirm the results of Abderhalden, but without success. Ordinarily he was unable to find lactase in the succus entericus also, but he stated that the enzyme could often be found after parenteral injections of lactose. The intestinal enzyme disappeared after ten days, in spite of continued introductions of the sugar. Another phase of the negative aspect has been contributed by Pincussohn and Krause (1914). They attempted to demonstrate sucrase in the blood after parenteral injections of sucrose, but with one exception they were unable to do so.

Considerable evidence has been accumulated, showing that glucose and fructose are utilized by the body tissues even when administered parenterally. Thus if a starving animal receives intravenous injections of those carbohydrates, the amount of glycogen in the liver increases. Ishimori (1913) confirmed this with rabbits, but did not obtain a similar result after administering lactose, maltose or galactose.

A later communication by Abderhalden (1914) indicates that the development of sucrase or a similar enzyme in the serum is more uncertain than the earlier papers indicated. He states here that some dogs do not develop the sucrase until after repeated injections. According to him, it may appear also after long continued feeding of sucrose. He believes that one difficulty in developing the enzyme in the serum is due to the rapidity of elimination.

In a recent paper, Kumagai (1914) confirmed Abderhalden's results, though he was unable to do so by the latter's method, in which the dosage was very small. According to Kumagai, a much more pronounced result is obtained by administering the sugar in large quantities, usually over 1 gram per kilo body weight. He also believes that the new factor is more than a mere sugar-splitting enzyme; it has the properties of an antigen and animals can be actively or passively immunized. Furthermore it exerts its action in a wholly unexpected way. If, for example, sucrose is subjected to the action of "immunized" serum, it becomes strongly levorotatory, and more so than mere inversion of the sugar could explain. Dextrose, when treated in a similar way, becomes levorotatory. If the products are allowed to stand long enough though, the optical behavior in either case will

become dextrorotatory again. When subjected to the same conditions, levulose will in time become dextrorotatory. Röhmann and Kumagai (1914) followed up their earlier observations, and have recently reported some very striking results. Kumagai had found that serum "immunized" to sucrose exerted a further action on its hydrolytic products. Dextrose was converted to levulose, and levulose was transformed into a disaccharide which these authors now claim to be lactose.

Abderhalden and his coworkers have still more recently returned to this problem; they have attempted in their later publications to confirm the findings of Kumagai, but their results were uniformly negative. In the first paper of this series (1914a) they investigated the relation of dextrose, levulose and galactose to blood serum, before and after parenteral administration of those sugars. In general their results were entirely negative, but minor exceptions are recorded. Another publication (1914b) is concerned with a similar study of sucrose; rabbits were the experimental animals in this series. The earlier work of Abderhalden (1910), which Kumagai was unable to confirm, was repeated. It was again demonstrated that the serum of normally nourished animals is unable to invert sucrose, but if the animals had received previous parenteral injections of that sugar, it would then be hydrolyzed by the blood. Such serum was entirely indifferent toward lactose, dextrose, levulose or galactose. It will be recalled that according to Kumagai this "activated" serum exerted a marked action on those sugars, as evidenced by their optical properties. These experiments were also repeated (1914c) with dogs, and Abderhalden confirmed his earlier work in every respect.

Even before the publication of these newer contributions to the question of developing in the blood carbohydrate-digesting enzymes absent from it under ordinary circumstances, the problem had been approached from a different angle. If this hypothetical exhibition of "protective enzymes" is to assume any physiological significance, it ought to enable the organism to utilize carbohydrates which are ordinarily wasted if introduced otherwise than by the oral path. In other words after repeated injections of sucrose or lactose, the sucrase and lactase postulated

as appearing in the blood stream ought to secure a sufficient conversion of the disaccharide into directly utilizable monosaccharides. Less and less of the parenterally introduced disaccharides should accordingly be lost to the body in the urine as the "immunization" of the animal proceeds. Already in an earlier publication of Mendel and Kleiner (1910) there is given an illustrative protocol of an experiment in which the utilization of parenterally introduced sucrose was not materially improved after twelve injections, repeated at frequent intervals. It is difficult to understand such failures if it be true that sucrase becomes abundant in the blood of an animal thus treated.

Leopold and Reuss (1909a) report more positive results in this field. Their method was to make repeated subcutaneous injections of lactose into dogs, and estimate the amount of sugar re-excreted in the urine. Many results are recorded indicating that 1 gram might be completely retained by a dog weighing a little over 4 kilos. A dog weighing 20 kilos was reported as retaining 6 grams on one occasion. A short time later (1909b) these investigators repeated the experiments with children, and again the results were positive. The authors claimed no especial advantage for their procedure however, because continued injection of any considerable amount of sugar proved toxic.

The consequences of improved utilization to be expected from repeated parenteral injections appeared so logical that at the suggestion of Professor Lafayette B. Mendel this line of investigation has been continued. The condition of the serum has not been taken into account in the present communication. In passing however several facts deserve to be recorded:

1. Although frequent attempts were made to demonstrate the appearance of sucrase or lactase in the blood under widely varied conditions of single or repeated parenteral administrations of corresponding sugars, all results were uniformly negative, despite the adoption of various methods of investigation.
2. The intraperitoneal method was commonly followed.
3. In this series no attempt has been made to demonstrate either the presence or absence of lactase in the intestinal mucosa, of the animals employed—a point on which Abderhalden has placed considerable emphasis.

EXPERIMENTAL.

The carbohydrates employed, lactose and sucrose, were introduced into dogs. Three of the animals were more or less continuously under observation for more than nine months, and the sugars were injected at frequent intervals. One animal, II, received approximately 170 injections during that time, and 125 of these were made within a period of less than six months. The administration of sugar was less frequent in the other two animals, averaging about three times per week. The dosage of the sugars injected intraperitoneally was usually 1 gram, in 8 per cent solution. Eight other dogs were under observation for shorter periods, ranging from a few days to over a month. Most of these received large doses for a portion of the time, because Kumagai was more successful with that method. In these cases the sugars were administered subcutaneously. If this method has the assumed efficacy in developing an enzyme or "antigen," then an animal should metabolize more sucrose after such treatment than before. In accordance with that hypothesis, small doses of sucrose were first given by the usual method to find the normal utilization. Following Kumagai, increased amounts were then given, and after that the former small dosage was repeated, to see if there was a greater tolerance for sucrose. The ordinary precautions were taken to preserve asepsis while making the injections, and no infection developed at any time. The diet contained a liberal allowance of meat, with free access to water.

For well known reasons exact quantitative determinations of sugar in urine are always more or less unsatisfactory. Preliminary trials indicated that the optical method was the most reliable, hence this was employed. Normal dog's urine usually contains substances that interfere with the accuracy of this method; therefore attempts were made to find a method of clarification that would remove with some degree of completeness the optically active compounds. A slight modification of a method suggested by Neuberg and Ishida (1911) was finally adopted as the most satisfactory. The advantages of the method are due to the combined use of mercuric acetate and phosphotungstic acid. In practically every case a normal urine treated in this way will give a zero polariscopic reading. Controls made

by adding known amounts of sugar to a definite volume of urine invariably yielded results correct within 0.1 per cent.

After the apparent utilization of the sugars introduced had been determined, it was deemed advisable to determine whether the sugar administered was excreted as such without undergoing any change. This seemed especially advisable in view of the recent publication from Röhmann's laboratory to which reference has already been made. Concerning this point it is only necessary to say that the results yielded positive evidence, in every case tested, that the sugar excreted was identical with the one introduced. Attempts were also made to demonstrate the presence of sucrase in the serum of some of the experimental animals, but all results were entirely negative.

It seems unnecessary to present in detail all the voluminous analytical data derived from these experiments. As an illustrative case, the protocols of dog II are published here somewhat elaborately. In the other cases briefer summaries are presented, the unessential details being omitted.

DOG II.

Weight, 9.8 - 13.8 Kilos.

DATE	SUGAR	AMOUNT IN- JECTED	AMOUNT EX- CRETED	DATE	SUGAR	AMOUNT IN- JECTED	AMOUNT EX- CRETED
		grams	grams			grams	grams
June 1913				1913			
9	Lactose	1.0	0.6	November			
11	Lactose	1.0	0.8	4, 5, 6, 7, 8,			
13, 16, 23, 24	Lactose	1.0	0.7	10, 11, 14,			
25, 30	Lactose	1.0		15, 16, 17,			
July				18, 19, 20,			
1			0.7	21, 22, 24,			
2	Lactose	1.0	0.6	25, 26, 27	Lactose	1.0	
4	Lactose	1.0	0.8	28			0.9
7	Lactose	1.0	0.9	29, 30	Lactose	1.0	
10, 13, 15, 17,				December			
18, 19, 20,				1, 2, 4, 5	Lactose	1.0	
21, 22, 23,				6			0.8
24	Lactose	1.0		6, 7, 8	Lactose	1.0	
25	Lactose	1.0	0.7	9	Lactose		0.4
27, 28, 29	Lactose	1.0		10, 11, 12	Lactose	1.0	
31	Lactose	1.0	0.9	13			0.8
				13, 15, 16,			

DOG II—Continued.

DATE	SUGAR	AMOUNT IN- JECTED	AMOUNT EX- CRETED	DATE	SUGAR	AMOUNT IN- JECTED	AMOUNT EX- CRETED
1913		grams	grams	December 1913		grams	grams
August				17	Lactose	1.0	
1	Lactose	1.0	0.8	18			0.9
2	Lactose	1.0	0.8	18, 19, 20,			
4	Lactose	1.0	0.7	21	Lactose	1.0	
5, 6, 7, 8, 12,				22			0.6
13, 14, 15,				January 1914			
16, 17, 18,				4, 6, 8, 10, 11,			
20, 21	Lactose	1.0		13, 15	Lactose	1.0	
September				16			0.9
2, 3, 4, 5, 6, 7,				19	Lactose	1.0	0.9
8, 9, 10, 11,				22, 24, 26,			
12, 13, 14	Lactose	1.0		28	Lactose	1.0	
15			0.8	29			0.9
16, 17, 18, 19,				30	Lactose	1.0	
23, 25	Lactose	1.0		February			
26			0.9	4, 6, 8	Lactose	1.0	
27, 28, 29	Lactose	1.0		9			0.9
October				10, 12, 14,			
1	Lactose	1.0	1.2	16, 18, 20,			
3	Lactose	1.0	0.8	22, 24, 26,			
8, 9, 10, 12,				28	Lactose	1.0	
13, 14, 15,				March			
16, 17, 18,				2, 4, 7, 9, 10	Lactose	1.0	
19, 20, 21,				12	Sucrose	3.2	2.5
22, 23	Lactose	1.0		14	Lactose	1.0	
24			0.7	16	Sucrose	3.4	3.0
25, 27	Lactose	1.0	0.6	17, 19, 21,			
29 30, 31	Lactose	1.0		22, 24, 26,			
November				28	Lactose	1.0	
1, 3	Lactose	1.0		April			
4			0.7	6	Lactose	1.0	0.9

The data concerning two other dogs, I, and II, differ in no essential respect from these recorded above.

In order to verify the accuracy of the analytical methods, and so confirm the findings concerning the degree of disappearance of parenterally introduced disaccharides, lactose and sucrose were administered to a few other animals, though the number of injections in each dog was far less extensive.

Dog IV, weighing 17 kilos, received 1 gram of lactose on July 4, and 1 gram on July 7. In each case 0.8 gram was recovered. Dog V, weighing 14 kilos, received 1 gram of sucrose on Nov. 5, and 0.3 gram was recovered. Dog VI, weighing 13 kilos, received 1 gram of sucrose on Dec. 8, 0.2 gram was recovered. On Dec. 12, the animal received 2 grams, and 1.1 grams were recovered. Dog VII, weighing 11 kilos, received on Jan. 15, 2.1 grams sucrose, 1.7 were recovered. On Jan. 19, the animal received subcutaneously, 11.3 grams sucrose, and 9 grams were recovered.

Additional experiments are briefly summarized below.

Dog No....	VIII	IX	X	XI	VIII	IX	X	XI	VIII	IX	X	XI
NO. OF INJECTION	NUMBER OF DAYS INTERVENING				GRAMS SUGAR INJECTED				GRAMS SUGAR EXCRETED			
					Sucrose	Sucrose	Sucrose	Sucrose		2.3		
1					1.0	3.2	3.2	3.3	0.7	2.3	1.7	
2	2	2	5	5	1.0	3.2	15.4	3.3	0.6	2.4	10.9	2.4
3	5	1	2	4	3.4	14.9	15.5	3.3	2.4	12.5		0.1
4	1	1	3	3	3.4	14.9	3.3	3.9	2.5	11.9	1.7	3.0
5	4	3	4	2	1.4	3.2	3.4	15.2	1.1	2.4	2.3	11.1
6	1	2		1	1.0	3.2		15.2	0.9	2.3		12.7
7				2				3.2				2.6
8				4				3.4				3.0
9				3				Lactose 3.4				3.1
10				3				Sucrose 3.5				2.5

Discussion of results. Concerning the results reported little need be said. Three of the dogs received intraperitoneal injections of lactose or sucrose throughout a period extending over several months. Except in some instances the dosage given was constant, and the quantity of sugar recovered was almost invariable. It is true that a very few exceptions appear: dog I on two occasions failed to excrete any of the lactose injected. In two or three other cases the amount recovered was considerably less than usual. Another dog, XI, presented an equally strange anomaly. In the latter case, 3.3 grams of sucrose were once administered, and practically none was recovered. In the face however of the mass of opposing data, and in view of the unavoidable difficulties in an experiment of this kind, it is not easy to find reason for believing that the abnormal results were due to the development of any unusual factor. The serum digestion trials also point

in the same direction, and if anything they are even more concordant.

It will be observed that the amount of sugar recovered varies within narrow limits from time to time, though the dosage was unchanged. Yet if these differences are to be accorded any significance, it is necessary to ignore all the interfering factors such as analytical difficulties. It may safely be said that no new biochemical reaction is necessary to explain such discrepancies. Some of the variations possibly are significant. When 1 gram of lactose was injected, the amount recovered ranged usually from 0.6 to 0.9 grams. If an equal amount of saccharose was administered, the amount recovered was uniformly less. It is probable that when this difference between the behavior of lactose and sucrose is explained, the reason will also be clear why complete recovery of either was so seldom attained.

In those trials where the dosage employed was large, there was again no evidence that a sucrase or lactase had been formed. The elimination of the foreign carbohydrate was as complete after the large doses as before. It should be remembered in this connection that Kumagai obtained the most active serum when he administered large doses of sucrose. In the experiments reported in this paper the serum digestion observations yielded no positive results. For the discrepancy between these findings and those of Kumagai, no explanation presents itself at present. It is interesting to note that Abderhalden, in contrast with the other worker, attained little success when large amounts of sugar were employed. Kumagai on the other hand was entirely unsuccessful with Abderhalden's method. Under the circumstances there is reason to believe that neither of them has solved the problem of the parenteral utilization of lactose and sucrose.

There is also every reason to believe that the sugar introduced was eliminated unchanged. When sucrose was injected, the urine would seldom reduce Benedict's solution. In the positive cases very slight hydrolysis combined with the normal reducing action of dog's urine may be sufficient to explain the result. The essential points are that in the trials made, fermentation tests with yeast were positive, and in general no osazone could be obtained in the untreated urine. The exceptions are easily explained, as sucrose is readily hydrolyzed with acetic acid, due to

the reagents employed, and then yields glucosazone. After hydrolysis glucosazone was obtained in large amounts; and most conclusive of all, the rotation after inversion agreed very closely with the theoretical value expected if the optically active substance were sucrose. The evidence is equally convincing concerning lactose. When it was introduced, the urine almost invariably reduced Benedict's solution, whereas fermentation tests with yeast were negative. In the cases where trials were made lactosazone was obtained. After hydrolysis with hydrochloric acid the rotation was increased, and mixtures of glucosazone and galactosazone were obtained.

Furthermore it seemed desirable to ascertain whether these dogs were normal in their sugar metabolism. To ascertain this, in a few instances the monosaccharides dextrose, galactose, and levulose were administered intraperitoneally. Insignificant amounts of the sugar were excreted after receiving galactose; but the uniformly negative results after administering the other monosaccharides amply verify the ability of the animals to utilize lactose or sucrose if they have once been hydrolyzed.

When viewed as a whole the data are in every way concordant; and *they yield no evidence whatever that any enzyme or any useful protective factor hitherto described is regularly developed in the animal body by the parenteral introduction of lactose or sucrose.*

SUMMARY.

Repeated injections of lactose and sucrose were made into the intraperitoneal cavity of dogs. Greater utilization of the sugars could not be attained by repeated introductions. "Immunity" was not developed.

The lactose and sucrose injected were excreted unchanged.

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SOME ANAPHYLACTIC REACTIONS.

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I. HAEMOGLOBIN SPECIFICITY.

The analyses of haemoglobins available in the literature, and the range of empirical formulae deduced, have never satisfactorily established the identity or the non-identity of haemoglobins of various sources. The limits of accuracy, the relatively small amount of iron in the molecule, and the uncertainties of purification have made this result inevitable. For ordinary chemical purposes all haemoglobins are alike and are identified by the same tests. Only in the crystal forms does there seem to be evidence of chemical difference, nor is this evidence at all conclusive since we have many examples of crystal dimorphisms of both elements and compounds. Such large differences of type and order as were found by Reichert¹ strongly suggest however that we have in different haemoglobins radically different molecular arrangements.

The anaphylactic reaction of the guinea pig was chosen therefore as a peculiarly delicate test of chemical identity or non-identity of haemoglobins. Rosenau and Anderson showed in their early work that washed corpuscles would sensitize,² and in the few cases tried were specific. Thomsen³ also showed that the crystallized haemoglobin of the dog and horse were quantitatively specific. The experiments recorded here therefore are merely an extension of the earlier work.

A series of guinea pigs was sensitized with beef haemoglobin freed from serum by washing the corpuscles twenty times and depositing

¹ Reichert and Brown: *The Crystallography of Hemoglobins*, 1909.

² Rosenau and Anderson: *Hygienic Laboratory Bulletin* No. 36. 1907.

³ Thomsen: *Zeitschr. f. Immunitätsl.*, iii, 1909.

in the centrifuge. The corpuscles were laked and no attempt made to remove the stroma. The solution was dried on glass at about 35° in a current of filtered air and kept in glass-stoppered bottles. A 20 per cent mixture of this material in water was ground thoroughly, filtered through cotton, and 0.5 cc. injected subcutaneously to sensitize.

Corpuscles from other sources were washed in a similar manner, laked and dried. An intoxicating dose of 5 cc. of the 20 per cent solution was injected intraperitoneally, ten days or more after sensitization. If the pig survived the injection of the second haemoglobin solution it was reinjected with the beef haemoglobin solution later. All of the pigs were thus found to be highly sensitive to the beef haemoglobin with but one exception. The results are recorded in Table I.

TABLE I*.

Guinea pigs sensitized with 0.5 cc. beef haemoglobin; Nos. 1-5, March 20; Nos. 6-9, May 7.

G. P. NO.	SECOND INJECTION OF 5 CC. INTRAPERITONEALLY								SYMPTOMS
	Chicken	Rabbit	Dog	Turtle	Pigeon	Goat	Sheep	Beef	
1								April 9	Severe; death
2	April 27							May 4	None.
3	April 27	April 30							Severe; death in 12 min.
4			April 25	April 27				May 4	None.
5		April 20	April 23					May 4	Severe; death.
6					May 24			May 2	None.
7						May 24		June 3	Severe; death in 15 min.
8						May 24		June 3	None.
9							June 3	May 21	Severe.
								June 3	Severe.

* These results were obtained by Mr. Jackman in this laboratory and appear in his graduating thesis.

The results show that eight of the nine sensitized animals were highly sensitive to beef haemoglobin, while none gave typical anaphylaxis with other haemoglobins. In the four cases terminating fatally autopsy showed a typical anaphylactic picture—lungs distended, the abdominal viscera much congested, and bloody fluid in the intestinal lumen.

It was felt that the above table did not accurately describe the experiment, in that mild or slight symptoms were ignored and only the typical severe symptoms of shock recorded as anaphylactic. It was also felt that the experiment was subject to criticism because pure haemoglobin, free from stroma material, had not been prepared. A second series was therefore undertaken in which pure crystallized dogs' haemoglobin was used for sensitization, and in which the dose was greatly reduced. Time of onset, severity of the symptoms and their duration, and the time required for recovery were taken as criteria of the reaction.

The preparation of crystalline dog haemoglobin was discovered by accident. It makes such an easy way of getting large quantities of crystals that it should prove of value in the class room and we therefore present it in detail here. Fresh, defibrinated dog's blood was mixed with 0.85 per cent sodium chloride solution and centrifugalized. The dilute serum was drawn off with a pipette and the corpuscles shaken up with more saline, and again centrifugalized. This process was repeated twenty times, when the corpuscles were laked with toluene and a little water. The toluene layer was removed and the solution centrifugalized and decanted from the stroma material. This haemoglobin solution was then mixed with 20 per cent by volume of toluene, shaken thoroughly and set aside in the cold. At the end of twenty-four or forty-eight hours the mixture is nearly a solid felt of large well formed crystals which may then be filtered off from the mother liquor, washed with a little cold water, spread thin on glass and dried either in *vacuo* or in a stream of warm filtered air. For demonstration purposes it is only necessary to mix defibrinated dog's blood with about 20 per cent toluene, shake and let stand in the cold overnight. The mass of crystals frequently becomes so solid that the flask may be inverted. On stirring up the mass it presents the silky sheen characteristic of suspended small crystals. Under the microscope it is seen to be a felt of the characteristic long prisms of dog's haemoglobin. We have not found other bloods which yield crystals with this treatment.

The haemoglobins required for second injection were not prepared so carefully as the sensitizing material. It is clear that if the sensitizing protein be perfectly free from the other blood pro-

teins—serum and stroma proteins—a specific reaction should only result from that portion of the second injection which represents haemoglobin. If the animal is not sensitized to serum or stroma, the injection of traces of serum or stroma with haemoglobin will not modify the result. The corpuscles of the various bloods used were therefore washed twenty times and centrifugalized, dried in warm filtered air and kept under sterile conditions. All haemoglobins were thus dried in order that strict comparisons might be made, using solutions of the same concentration throughout the series. For use the haemoglobins were dissolved in normal saline to a strength of 3.75 per cent. Enough formalin was added to make a 0.25 per cent solution to insure sterility. Schittenhelm⁴ has shown that much larger amounts of formalin produce no symptoms on injection, and the specificity of the protein molecule for anaphylaxis is not altered.

The results are collected in Table II.

From the results tabulated it is clear that the haemoglobin molecule is quantitatively specific for each species, and that the specificity is of a rather marked order, comparing very favorably with that between proteins apparently much more unlike. Of the sixteen different haemoglobins reinjected, eleven gave practically no symptoms, four showed slight atypical symptoms, while two were clearly anaphylaxis of only moderate severity. In every case where the sensitizing haemoglobin was reinjected the reaction was typical and severe. It may readily be made fatal by increasing the second injection.

It is interesting to note that guinea-pigs sensitized to dogs' haemoglobin reacted mildly to pig, turtle, and cat, but not to the strictly herbivorous haemoglobins nor to human haemoglobins. Dog serum produced noteworthy reactions in the two trials. Both were quite strongly tinged with haemoglobin however so that the interpretation is uncertain. It is possible that there was enough haemoglobin present to account for the reaction, though this seems unlikely. On the other hand it is quite possible that dogs' serum proteins and dogs' haemoglobin have a molecular structure of the protein molecules much alike, so that sensitizing with one renders sensitive to several of the blood components. If we assume that

⁴ Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, xi, 1912. Also Rosenau and Anderson: *loc. cit.*

TABLE II.

Sensitized with 0.5 cc. 3.75 per cent dogs' haemoglobin, March 26.

NO. G.P.	DATE	MATERIAL*	RESP. SPASM	PARALYSIS	PROSTRATION	RECOVERY COMPLETE
2	4/30	Dog	Severe	Severe	Very severe	After 12 hrs.
2	5/24	Turtle	None	Medium	Severe	In 49 min.
3	4/30	Pig	Medium	Medium	Medium	In 2 hrs.
3	5/23	Dog	Severe	Severe	Severe	Very sick at end of 6 hrs. killed.
4	5/24	Chicken	None	None	None	Normal
5	4/30	Calf	None	None	None	Normal
5	5/24	Turtle	None	Slight	Slight	In 1 hr.
7	4/30	Horse	None	None	None	Normal
7	5/24	Turtle	None	Slight	Slight	In 1 hr.
9	5/24	Goat	None	None	None	Normal
10	5/24	Dog	Severe	Severe	Severe	In 24 hrs.
11	5/24	Pig	None	Slight	Slight	In ½ hr.
13	5/24	Serum (dog)	Slight	Slight	Medium	In 2 hrs.
14	4/30	Cat	Medium	Slight	Slight	In 1½ hrs.
14	5/24	Serum (dog)	Severe	Severe	Medium	In 7 hrs.
15	4/30	Rabbit	Slight	None	Slight	In 1 hr.
15	5/24	Rat	None	None	Slight	In 1 hr.
16	5/24	Guinea P.	None	None	None	Normal
17	4/30	Sheep	None	None	None	Normal
17	5/24	Human	None	None	None	Normal

* All guinea pigs were reinjected with 5 cc. of a 3.75 per cent solution of the haemoglobin to be tested.

the relatively simple haematin portion of the molecule is the same in all haemoglobins, the specific differences must be found in the protein or "globin" fraction. In the ordinary acid cleavage of haemoglobin however these differences which render haemoglobins specific, are lost, for globin so prepared has been shown to be non-specific, though toxic itself.⁵

⁵ Schittenhelm: *Munch. med. Woch.* lix. 1912.

II. ISOGENOUS ANAPHYLAXIS

The sensitization of an animal to his own tissue-proteins or to his species-proteins is still a moot question. Rosenau and Anderson⁶ sensitized guinea pigs to guinea pig placental proteins, and the work of Abderhalden⁷ in developing serum diagnosis for pregnancy depends upon this fact. This latter work has been abundantly confirmed in medical practice. In terms of Vaughan's⁸ hypothesis this would seem to imply an escape into the general blood supply of proteins peculiar to the placenta—foreign therefore to the blood and to the other tissue cells—with a resulting development of enzymes specific for the placental proteins. Ordinarily so little placental protein gets into the blood that no toxic symptoms result. In eclampsia however, as Rosenau and Anderson pointed out, we have definite anaphylactic symptoms very possibly due to rapid cleavage of placental proteins. On the other hand injection of an organ extract does not seem to lead to definite degeneration of the homologous organ in the animal and this may be interpreted to mean that no specific proteolytic enzymes were produced. It may very well be however that specific enzymes, though present in the blood, would produce no digestion of the specific proteins fixed in the tissue cells and separated from the blood by one or more membranes.

The point of view that an animal's tissue proteins may act like foreign proteins, in developing protective enzymes of more or less specificity, when introduced into the blood stream appeared attractive enough to warrant further investigation, despite the doubtful or negative results already found.

Method. Organs were removed under aseptic conditions from a freshly killed guinea-pig. The tissues were weighed, ground fine and mixed with sufficient normal sterile saline to make 10 per cent tissue extracts. Shreds of connective tissue were removed by straining and sufficient formalin added to give a final strength of 0.25 per cent. The turbid tissue extracts were used at once for sensitization, 0.5 cc. subcutaneously being injected. Ten days or more later the pigs were reinjected with 5 cc. of the same turbid

⁶ Rosenau and Anderson: *loc. cit.*

⁷ Abderhalden: *Schutzfermente des tierischen Organismus*, 1912.

⁸ Vaughan: *Journ. Amer. Med. Assoc.*, xlvii, p. 1009.

extracts intraperitoneally. In other cases the pigs were reinjected with 5 cc. of freshly prepared tissue extracts. The results were indistinguishable so that the formalin appeared to have checked autolysis completely without having destroyed the potential toxicity of the molecule.

The results of the first series are summarized in Table III.

TABLE III.

NO.	MATERIAL	RESP. SPASM	PARALYSIS	PROSTRA- TION	RECOVERY
1	1. Sem. vesicle 2. Liver	Severe	Medium	Medium	In 2 hrs.
2	1. Liver 2. Liver	Severe	Medium	Medium	In 2 hrs.
3	1. Muscle 2. Muscle	Severe	Medium	Medium	In 2 hrs.
4	1. Testicle 2. Testicle	Severe	Severe	Severe	In 2 hrs.
5	1. Heart 2. Heart	Severe	Severe	Medium	In 3 hrs.
6	1. Lung 2. Lung	None	Medium	Medium	In 1½ hrs.
7	1. Kidney 2. Kidney	Medium	Medium	Slight	In 1½ hrs.
8	1. Control 2. Muscle	Medium	None	None	In 1 hr.

Note.—0.5 cc. used to sensitize; 2 or 2.5 cc. to intoxicate.

The results obtained with this group of young animals, from 160 to 445 grams, weight, are not very conclusive. The sensitized animals showed typical symptoms of shock, while the control did not. In the single case of cross injection the symptoms were quite as severe as in reinjection. No definite specificity could therefore be assumed. On the whole the previously injected animals all showed more severe intoxication than the normal animal. This suggests sensitization.

Series II was better controlled than the preliminary group. The results are summarized in Table IV.

The three controls, injected with liver extract, all showed slight intoxication, with mild paralysis and prostration, and with no respiratory spasms characteristic of typical anaphylaxis, when at all severe. On the other hand all of the sensitized animals showed

TABLE IV.

NO.	MATERIAL		RESP. SPASM	PARALYSIS	PROSTRA- TION	RECOVERY
1.	1. Control					
2.	2. Liver	5cc.	None	Slight	Slight	In 3 hrs.
3.	1.*Egg albumin					
2.	2. Egg albumin	5cc.	Medium	Severe	Severe	In 18 hrs.
3.	1. Liver					
2.	2. Liver	5cc.	Slight	Severe	Severe	In 12 hrs.
4.	1. Liver					
2.	2. Liver	7cc.	Slight	Slight	Slight	In 12 hrs.
5.	1. Muscle					
2.	2. Muscle	5cc.	Medium	Severe	Severe	In 12 hrs.
6.	1. Muscle					
2.	2. Muscle	7cc.	Severe	Medium	Severe	In 12 hrs.
7.	1. Heart					
2.	2. Heart	5cc.	Severe	Severe	Severe	Died in 8 hrs. No peritonitis.
8.	1. Heart					
2.	2. Heart	6cc.	Severe	Severe	Severe	Died before 12 hrs. No peritonitis.
9.	1. Sem. vesicle					
2.	2. Testicle	5cc.	None	None	None	Normal
10.	1. Sem. vesicle					
2.	2. Sem. vesicle	5cc.	Severe	Severe	Severe	In 12 hrs.
11.	1. Testicle					
2.	2. Testicle	5cc.	Severe	Severe	Severe	Died before 12 hrs. Puncture peritonitis.
12.	1. Testicle					
2.	2. Testicle	7cc.	Severe	Severe	Severe	In 18 hrs.
13.	1. Control					
2.	2. Liver	7cc.	None	Slight	Slight	In 6 hrs.
14.	1. Control					
2.	2. Liver	7cc.	None	Medium	Slight	In 3 hrs.

* Pigs were sensitized with 0.5 cc. of the designated extract (1).

intoxication with severe symptoms in the majority of cases. All showed respiratory spasms, most of them severely; all were paralyzed and all were prostrated after the acute symptoms were over. All but one were severely prostrated, remaining sick many hours, and of these two died of anaphylactic shock. In the single case of cross-injection there were no symptoms at all. Here again the experiment as a whole seems to indicate a certain amount of sensitization as a result of the preliminary injection.

A third series was observed with still more rigid control. The normal and the previously injected pig were given the intoxicating dose at the same moment, placed side by side in the observation cage and their symptoms recorded with the time of onset and duration and their comparative severity. The results are collected in Table V, and summarized in Table VI.

TABLE V.

NO.	MATERIAL	RESP. SPASM	PARALYSIS	PROSTRA- TION	RECOVERY
1	Liver	None	Medium	Medium	In 24 hrs.
2	Control	None	Medium	Slight	18 hrs.
3	Muscle	Medium	Severe	Severe	In 36 hrs.
4	Control	None	Slight	Slight	Less than 24 hrs.
5	Muscle	Severe	Severe	Severe	In 24 hrs.
6	Control	None	Medium	Medium	In 2 hrs.
7	Muscle	None	Slight	Slight	In 4 hrs.
8	Muscle	Severe	Severe	Severe	In 4 hrs.
9	Kidney	Severe	Severe	Very severe	In 24 hrs.
10	Control	None	Medium	Medium	In 2 hrs.
11	Kidney	Medium	Medium	Medium	In 24 hrs.
12	Sem. vesicle	Severe	Medium	Severe	In 24 hrs.
13	Control	Severe	Medium	Severe	In 6 hrs.
14	Liver	None	Medium	Medium	In 4 hrs.
15	Control	None	None	Slight	In 1 hr.
16	Heart	Severe	Severe	Severe	In 24 hrs.
17	Control	Slight	Medium	Medium	In 4 hrs.
18	Muscle	Severe	Severe	Severe	In 12 hrs.
19	Muscle	Medium	Severe	Severe	In 12 hrs.
20	Control	None	None	Very slight	In 3 hrs.

Note.—Pigs were sensitized with 0.2 cc.; reinjected with 5 cc. in each case. The control was injected with 5 cc. of the same material used in the test, without previous injection.

From the summary it appears quite clear that the normal guinea pigs react to the injection of guinea-pig tissue-proteins less severely than those previously injected. In only one of the eight controls was the respiratory spasm severe; it was present in only two. On the other hand the previously injected animals showed the spasms in 75 per cent and in 50 per cent it was severe.

The degree of paralysis of the normal animals was less severe than in the test pigs, none of the controls were as severely paralyzed as

TABLE VI.

	RESP. SPASM		PARALYSIS		PROSTRATION	
8 Controls	None	6	None	1	None	0
	Slight	1	Slight	2	Slight	4
	Medium	0	Medium	5	Medium	3
	Severe	1	Severe	0	Severe	1
12 Sensitized	None	3	None	0	None	0
	Slight	0	Slight	1	Slight	1
	Medium	3	Medium	4	Medium	3
	Severe	6	Severe	7	Severe	8

were 50 per cent of the test pigs. The prostration of the control pigs was also much less marked than the test animals, and the time required to return to normal was as a rule less. There are however a number of exceptions, and the criteria themselves—representing quantitative differences judged only by the eye—are unsatisfactory. We believe however the data warrant the assumption that tissue proteins present in the blood stream do cause immunity reactions of a rather low order; that tissue proteins are essentially foreign when present in the blood stream and lead to a sensitization so that reinjection of the same material leads to symptoms of intoxication quantitatively more pronounced than in the normal animal. All the experiments confirm the observation that tissue extracts are quite toxic to the same species.⁹ Whether this is due to a slight normal sensitization of the animal by occasional escape of traces of tissue proteins into the circulation, or whether it is due to small amounts of protein cleavage products in the organs used cannot be determined from these experiments.

CONCLUSIONS.

I. Guinea pigs sensitized to beef or dog haemoglobin fail to react, or react but slightly, to haemoglobins of other origins. The haemoglobins tried were dog, beef, cat, rabbit, rat, turtle, pig, horse, calf, goat, sheep, pigeon, chicken, and man.

II. Haemoglobins from different sources are chemically different.

III. A low order of sensitization to isogenous proteins is found in guinea pigs injected with guinea-pig tissue-proteins.

⁹Bauer and Wüsthof: *Deutsch. med. Wochenschr.*, xxxviii, 1912.

THE BLOOD IN ACIDOSIS FROM THE QUANTITATIVE STANDPOINT.

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In his discussion of diabetic acidosis, Magnus-Levy¹ expressed the view that the "acid poisoned animal and the diabetic patient do not die from the acid which has been *eliminated* in a neutralized state, but from the acid which *remains in the body*." But little is known as to just what extent acetoacetic acid and oxybutyric acid may accumulate in the blood and tissues, for only a few analyses of the blood and organs of those dead of acidosis have been published. Many of these are of questionable accuracy, and certainly inaccurate as far as oxybutyric acid is concerned, as determinations were made by means of polariscopic methods² and we know that optically active substances other than oxybutyric acid may occur in blood and tissue extracts. In the more recent determinations of Sassa,³ analytical procedures capable of greater accuracy were made use of, namely the Shaffer oxidation method. The analyses of human tissue were, however, made on material obtained at autopsy some thirteen to twenty-seven hours after death, and it is conceivable that some changes could have occurred during this interval. As far as I can find no determinations of the acetone bodies in blood taken directly from the living human subject, who alone appears to exhibit extensive oxybutyric acid acidosis, have been made,⁴ so that but little is definitely known as to how much

¹ Magnus-Levy: *Johns Hopkins Hospital Bulletin*, xxii, p. 46, 1911.

² Cf. Magnus-Levy: *Arch. f. exp. Path. u. Pharm.* xlii, p. 149, 1889.

Minkowski: Quoted in Waldvogel, *Die Acetonkörper*, p. 61.

Hugounenq: *Ref. Jahresber. d. Tierchem.*, xvii, p. 430, 1887.

³ Sassa: *Biochem. Zeitschr.*, lix, p. 362, 1914.

⁴ A few preliminary determinations were published in a preceding article. Marriott: *This Journal* xvi, p. 293, 1913.

acetoacetic acid and oxybutyric acid may be present in the body during life.

A method which was recently published⁵ makes it possible to determine the acetone bodies in small samples of blood (1-5cc.). This method has since been simplified and at the same time rendered more accurate. With the improved technique, a determination may be carried through in about four hours. A number of blood analyses may be run at the same time so that a half dozen complete analyses may be completed in a day.

Analytical methods.

Blood is drawn by venous puncture or, as in the case of infants, by cupping.⁶ If there is an appreciable acidosis, as shown by a urine test, 1 or 2 cc. of blood is ample for a determination. Larger amounts of blood, up to 5 cc., may be used in cases where there is little or no acidosis. The blood is run into a weighed 50-cc. Erlenmeyer flask containing 20 cc. of 0.1 per cent potassium oxalate.⁷ The flask is again weighed and, from the increase in weight, the quantity of blood is determined.

The blood proteins are precipitated by the colloidal iron method of Michaelis and Rona,⁸ as follows: sufficient colloidal iron⁹ solution is run into the flask to bring the total volume to 50 cc., the contents are mixed and then about 0.5 grams of powdered sodium sulphate is added, the flask is stoppered and vigorously shaken to dissolve the sodium sulphate and to break up the jelly-like coagulum formed.¹⁰ The thick liquid is poured into a 50-cc.¹¹ centri-

⁵ Marriott: *loc. cit.*

⁶ Some preformed acetone is likely to be removed from the blood by the vacuum produced in cupping.

⁷ In order to prevent decomposition sterile solutions are advised, and the flask contents should be cooled on ice before blood is drawn and kept in the ice box until used.

⁸ Michaelis and Rona: *Biochem. Zeitschr.*, vii, p. 329, 1908.

⁹ Merck's "Iron dialyzed," 5 per cent Fe_2O_3 , was used.

¹⁰ The coagulum should be brown. If it is reddish, insufficient iron solution has been added. In such a case the liquid is poured into a beaker and 25 cubic centimeters more of iron solution added and the whole stirred vigorously.

¹¹ If such tubes are not available, the liquid may be put in three ordinary 15 cc. tubes.

fuge tube, and centrifugated for a couple of minutes, the clear supernatant liquid is poured off through a filter paper and used for the determination.

Combined determination of acetone and acetoacetic acid.

An aliquot portion (usually 15 or 30 cc.) of the protein-free filtrate is measured into an 800-cc. Kjeldahl flask. About 500 cc. of water, a little talc, and 30 cc. of sulphuric acid (sp. gr., 1.59) are added. The flask is connected up to a Kjeldahl still and distilled for twenty minutes after boiling has begun. The residue in the flask is used for oxybutyric acid determination, details of which are given below. The distillate which contains acetone, preformed and from acetoacetic acid, is collected in a 500-cc. Kjeldahl flask containing about 100 cc. of water.¹² In very warm weather it is advisable to cool the receiving flask by surrounding it with cracked ice. The distillate is redistilled after the addition of 5 cc. of 3 per cent hydrogen peroxide, and 5 cc. of 10 per cent sodium hydroxide and a little powdered talc. The flame should be very low at the beginning to permit complete decomposition of aldehydes or sulphides by the peroxide before distillation begins. If this is done, it is quite unnecessary to resort to heating with a reflux condenser, as suggested by Mondscheim.¹³ The distillate is caught in a 100-cc. Erlenmeyer flask having a graduation mark at exactly 100 cc., and containing 25 cc. of "Acetone Reagent" prepared according to Scott-Wilson.¹⁴ Distillation is continued until the 100 cc. mark is reached. While this distillation is going on, known amounts of acetone¹⁵ are distilled into Erlenmeyer flasks containing the re-

¹² In all distillations of acetone the tip of the delivery tube must dip under the surface of liquid in the receiving flask.

¹³ Mondscheim: *Biochem. Zeitschr.*; xlii, p. 100, 1912.

¹⁴ Scott-Wilson: *Journ. of Physiol.* xlii, p. 444, 1911. 10 grams of mercuric cyanide and 180 grams of sodium hydroxide are separately dissolved in water, the solutions poured together and the whole made up to 1200 cc. To this is slowly added with stirring a solution of 2.9 grams of silver nitrate in 400 cc. of water.

¹⁵ A stock solution of acetone is prepared from pure acetone and its value determined by titration of 200 cc. by the Messinger method. A convenient strength for a standard solution is 0.02 to 0.03 mgm. per cc. Usually a 3-cc. or a 5-cc. portion of this stock solution is distilled into the reagent. Such turbidities as are occasioned approximate those produced by blood distillates.

agent as above. After standing for at least twenty minutes, the turbidities in the different flasks are compared in the nephelometer of Richards,¹⁶ first diluting the standards or unknown solutions, if necessary, to obtain turbidities approximately equal.¹⁷ For fuller details concerning the use of the nephelometer, the reader is referred to a previous communication.¹⁸

Separate determination of preformed acetone and of acetoacetic acid.

While ordinarily one wishes to determine only "gross acetoacetic acid," i.e., acetone plus acetoacetic acid, occasionally the separate determination is desired. In order to do this, an aliquot portion (15 cc.) of the filtrate, after the colloidal iron precipitation, is transferred to a small cylinder (200×35 mm.) and a rapid current of air drawn through to remove preformed acetone. Using a small brass water pump, and drawing air into the cylinder through a small Folin ammonia absorption tube,¹⁹ the preformed acetone is removed within twenty minutes. The liquid in the cylinder is then transferred to an 800-cc. Kjeldahl flask, 500 cc. of water and 30 cc. of sulphuric acid (sp. gr., 1.59) are added and the distillation and determination carried out exactly as described above. In this case the acetone in the distillate is from acetoacetic acid only, so that by difference the amount of preformed acetone previously present can be calculated.

¹⁶ Richards: *Zeitschr. f. anorg. Chem.*, viii, p. 269, 1895. Richards and Wells: *Amer. Chem. Journ.*, xxxi, p. 235, 1901. A nephelometer is necessary as an ordinary colorimeter of the Duboseq type cannot be used for a comparison of such faint turbidities as are obtained from the amounts of blood used.

¹⁷ For accurate results it is essential that the solutions compared shall differ by not more than 20 or 25 per cent from each other. Such differences can readily be detected by the eye before placing the solutions in the nephelometer.

¹⁸ Marriott: this *Journal* xvi, p. 289, 1913. In this paper, on page 291, I stated that if sufficient depths of solution were used it was unnecessary to apply a correction, and that Kober's equation for a correction curve indicated that with greater depths of solution the correction was proportionately less. Kober, in a private communication, has kindly drawn my attention to the fact that I have misinterpreted his equation. The observed facts are correct as stated, but are not to be accounted for by Kober's equation.

¹⁹ Folin: this *Journal* xi, p. 499, 1912.

Determination of oxybutyric acid.

The liquid remaining in the 800-cc. Kjeldahl flask, after distilling off acetone, is used for the determination of oxybutyric acid. The receiving flask is changed, a liter Kjeldahl flask being substituted. It is not necessary to stop the boiling while the change is being made. The receiving flask should contain about 100 cc. of water.

Through a dropping funnel a 0.1 per cent solution of potassium bichromate is run into the distilling flask at such a rate that the volume of solution remains between 400 and 500 cc. Usually 50-cc. portions of the bichromate solution are added at a time, the flame being raised while the solution is being run in, to prevent cooling of the liquid to such an extent that negative pressure is developed sufficient to cause a back flow from the receiving flask.²⁰ In the intervals a low flame is used—just sufficient to keep the liquid boiling. Distillation is continued for two hours. During the first hour and a half, 300 cc. of bichromate are added, and during the last half hour, 200 cc. The latter part of the distillation may well be more rapid. Acetone in the distillate, the product of the oxidation of oxybutyric acid, is determined exactly as described above, after redistillation over peroxide and alkali, the distillate being caught in "acetone reagent." Oxybutyric acid, when oxidized with bichromate, yields only about 90 per cent of the theoretically obtainable acetone.²¹ It was thought that perhaps very small amounts of oxybutyric acid would be quantitatively converted into acetone by the relatively large excess of bichromate present, but this appears not to be the case. After making the correction for the blank reagents noted below, the results are from 90 to 95 per cent of the theory.

In carrying out the method using only the reagents some turbidity is always occasioned in the acetone reagent in which the final distillate is caught. I have been unable to determine the nature of the substance causing the turbidity. The amount of turbidity, however, is constant for any given reagents, so that having

²⁰ If the liquid in the receiving flask does "suck back" the determination is not necessarily lost, as the acetone may be again distilled off.

²¹ Shafer and Marriott: this *Journal* xvi, p. 265, 1913.

once determined this "blank" value it may be subtracted from the amounts actually found.²²

The following analyses of blood from a normal dog with and without the addition of oxybutyric acid ²³ show the character of results obtained. The "blank" values were subtracted and 10 per cent was added to the final result. The amounts of oxybutyric acid are expressed in terms of milligrams of acetone per 100 grams of blood.

Blood alone.....	0.00
Oxybutyric acid added.....	7.1
Oxybutyric acid found.....	7.5
Oxybutyric acid added.....	11.8
Oxybutyric acid found.....	12.5
Oxybutyric acid added.....	23.6
Oxybutyric acid found.....	24.8

With the 10 per cent correction the results are 5 per cent too high.

The question may be raised whether the turbidity in the acetone reagent may not be caused by substances other than acetone or oxybutyric acid present in the blood. In this connection it may be stated that the distillates from normal blood give only the slightest turbidity, whereas distillates from the bloods of subjects of acidosis give heavy turbidities in the reagent. I have found, further, that glucose, lactic acid, and ethyl alcohol in such maximum amounts as may exist in a 5-cc. sample of blood, when subjected to the analytical procedure, give distillates causing only exceedingly slight turbidities. For example, 40 mgm. of glucose, 10 mgm. of lactic acid and 10 mgm. of alcohol, gave in the final distillates turbidities equivalent to those produced by 0.01 mgm., 0.03 mgm. and 0.01 mgm. of acetone respectively.

²² In the reagents which I used, the "blank" for the determination of acetone and acetoacetic acid amounted to 0.025 mgm. of acetone, and for oxybutyric acid 0.035 mgm. acetone.

²³ A solution of *l*-oxybutyric acid purified by means of calcium zinc double salt was used. Its strength was determined by the polariscope and then it was suitably diluted and portions of the dilute solution measured out and added to the blood.

Applying the analytical methods to blood taken from approximately normal subjects, the results appearing in Table I were obtained.

TABLE I.

Analysis of the blood in conditions not associated with acidosis. Results calculated in terms of acetone and expressed as milligrams per 100 grams of blood.

	Acetone and acetoacetic acid	Oxy- butyric acid
1 Normal adult.....	0.0	0.0
2 Normal adult.....	0.0	0.0
3 Normal adult.....	0.0	0.0
4 Normal adult.....	0.7	2.1
5 Normal adult.....	0.0	2.1
6 Adult—3 hours after ether anaesthesia.....	0.8	2.0
7 Adult—advanced tuberculosis.....	0.9	2.0
8 Adult—pleurisy, temp. 101°.....	0.7	1.9
9 Adult—cerebrospinal lues, febrile.....	1.3	2.0
10 Adult—ascites, cirrhosis of the liver.....	1.2	4.0
11 Child—5 yrs. old, normal.....	0.6	4.4
12 Child—7 yrs. old, normal.....	0.8	4.4
13 Child—6 yrs old, tuberculous hip.....	1.1	1.8
14 Child—8 yrs. old, normal.....	0.5	1.4
15 Child—10 yrs. old, normal.....	0.6	2.8
16 Child—10 yrs. old, normal.....	0.5	2.8
17 Child—Normal.....	0.7	2.8
18 Dog—normal.....	0.6	2.1
19 Dog—normal.....	0.6	2.2
20 Dog—normal.....	0.6	0.3
21 Dog—starved 3 days.....	0.7	1.6
22 Pig—normal.....	0.4	0.5
23 Pig—normal.....	0.3	0.6
24 Pig—normal.....	0.1	0.6
25 Beef blood—defibrinated..	0.8	3.0

Separate determinations of preformed acetone and of acetoacetic were made in the blood of a number of these subjects with the result that, within the limits of error of the method, no preformed acetone was found to be present. The amounts of acetoacetic acid and of oxybutyric acid were, in all cases, very small. Normal blood contains less than 1.5 mgm. of acetoacetic acid and less than 4 mgm. of oxybutyric acid in 100 grams of blood; the results being calculated in terms of acetone.

In conditions associated with acidosis the blood determinations invariably give results departing considerably from the normal, as is seen in Table II.

The highest figures obtained for the total acetone bodies (No. 6) were from a case of diabetes with signs of impending coma. From the evidence at present in hand it cannot be stated that coma necessarily ensues when the acetone bodies reach a certain concentration in the blood.

Neubauer²⁴ has claimed that, when large amounts of acetone bodies are excreted in the urine, oxybutyric acid constitutes a larger proportion of the total than when the total excretion is smaller. The percentage of the total acetone bodies which oxybutyric acid constitutes, when all of the substances are expressed as acetone, has been referred to by Kennaway, Pembrey and Poulton²⁵ as the β ratio. This ratio has been found by them to be, in general, higher with increased total acetone-body output, this finding being in confirmation of Neubauer's results. The connection, however, between high β ratio and increased acetone body output was not by any means close. As will be seen from the table, my results on the blood have shown no connection whatever between the β ratio and concentration of acetone bodies present. The significance of the β ratio is not at present evident.

The effect of alkali therapy on diabetic acidosis is shown in the results obtained from the severe diabetic *K* (Nos. 6, 7, 8, in the table). The effect of the alkali was apparently to remove acetone bodies from the blood and, at the same time, to diminish the decomposition of acetoacetic acid into acetone.

In the blood, in conditions of acidosis, a large amount of the acetoacetic acid present may be converted into acetone. In Nos. 6 and 8 in the table preformed acetone made up more than 60 per cent of the gross acetoacetic acid, a figure much higher than would have been expected from any hitherto reported urinary analyses²⁶ in which the preformed acetone rarely amounts to as much as 10 per cent of the gross acetoacetic acid. This makes it evident

²⁴ Neubauer: *Verhand. d. d. Kong. f. inn. Med.*, xxvii, p. 566, 1910.

²⁵ Kennaway, Pembrey and Poulton: *Journ. of Physiol.*, xlvii, p. x. (Proc.) 1913.

²⁶ Cf. Embden and Schliepp: *Centralbl. f. d. ges. Physiol. u. Pathol. d. Stoffw.*, (N. F.) ii, p. 250 and 289, 1907. Folin: this *Journal*, iii, pp. 177, 1907.

TABLE II.

Analysis of the blood in conditions associated with acidosis. Results calculated in terms of acetone and expressed as milligrams per 100 grams of blood.

NO.	SUBJECT	ACETONE AND ACETOACETIC ACID	OXYBUTYRIC ACID	TOTAL ACETONE BODIES	β RATIO	REMARKS
1	Light diabetic	3.0	7.1	10.1	70	Urine passed at time blood was drawn contained acetone and acetoacetic acid 1.7 mgm., oxybutyric acid 5.8 mgm. per 100 grams.
2	Light diabetic	1.6	6.2	7.8	79	Urine passed at same time contained acetone and acetoacetic acid 1.0 mgm., oxybutyric acid 6.9 mgm. per 100 grams.
3	Light diabetic	11.9	13.1	25.0	52	
4	Severe diabetic	28.2	37.4	65.6	58	
5	"K" severe diabetic March 30	11.2	18.5	29.7	62	Carbohydrate free diet.
6	"K" severe diabetic April 16	28.0	45.2	73.2	62	Preformed acetone 17.6 mgm. per 100 grams = 63% of gross acetoacetic. Patient showed signs of impending coma. Total acetone bodies in urine for 24 hours 56.7 grams (expressed as acetone) β ratio 82. Carbohydrate free diet.
7	"K" severe diabetic April 24	11.0	13.5	24.5	55	Preformed acetone 2.8 (expressed as acetone) 15.9 grams β ratio 77. Daily doses of 40 grams sodium bicarbonate for preceding week. 50 grams of starch added to diet.
8	"K" severe diabetic May 8	18.0	49.5	67.4	73	Preformed acetone 11.0 mgm. per 100 grams = 61% of gross acetoacetic. Total acetone bodies in urine for 24 hours (expressed as acetone) 22.1 grams. β ratio 78. Mixed diet. No alkali for several days preceding.
9	Child in coma	23.4	24.8	48.2	52	Cerebral malaria—Inanition.
10	Child—post operative acidosis	11.2	28.0	39.2	71	Blood sample taken 24 hours after orthopedic operation.
*11	Infant—15 mo. Exudative diathesis	3.3	6.6	9.9	67	Preformed acetone 0.0.
*12	Infant—2 yrs. Pyelitis, infected sudamina, syphilis	13.3	15.8	29.1	54	Preformed acetone 3.8 mgm. per 100 gram = 28% of gross acetoacetic. No symptoms of acidosis.
13	Dog—coma	8.0	24.8	32.8	76	Dog injected subcutaneously with 25 grams of sodium butyrate.
14	Dog—phlorhizin diabetes.	7.6	14.0	21.6	65	Animal starved 4 days. Injections of phlorhizin daily.

* For these two analyses, I am indebted to Dr. Richard S. Weiss, of the Department of Dermatology.

that acetone itself must be excreted with difficulty by the kidneys. It is readily excreted by the lungs, however, as is shown by an analysis of the breath of the same patient.

The subject was made to inhale through his nose and exhale through a rubber tube connected with two Woulff bottles in series, each of which was fitted with a dropping funnel. Each bottle contained 100 cc. of water and 10 cc. of 60 per cent sodium hydroxide. Just before the experiment was begun, 25 cc. of $\frac{N}{10}$ iodine solution was added to the first bottle and 10 cc. to the second bottle. 25 cc. of $\frac{N}{10}$ iodine solution was measured into each dropping funnel. The patient breathed through the bottles for five minute periods during which time the iodine solution from the dropping funnels was slowly run in. The patient's respirations were of the same frequency as when breathing free, and of approximately the same depth. An abundant iodoform precipitate was produced in the first bottle and a smaller amount in the second. At the end of each five-minute period the contents of the Woulff bottles were transferred to a flask, acidified and titrated with thio-sulphate according to the usual Messinger method.²⁷

A five-minute respiration period at 5 p.m., April 15, and a second five-minute period the following day at noon gave practically identical results, which when calculated on the basis of twenty-four hours, indicated an excretion by the breath of 3.3 grams of acetone. During the same twenty-four hour period approximately 10 grams of gross acetoacetic acid, corresponding to less than 1 gram of preformed acetone, were excreted in the urino.

The figures for acetone bodies in the blood do not of course tell how these substances are distributed in the tissues. The following experiment indicates, in a general way, what the relative concentrations of the acetone substances in the various organs may be.

A dog was phlorhizinized and fasted for four days, killed, and the blood and tissues analyzed for the acetone bodies with the results shown in Table III.

The concentration of total acetone bodies in the liver and blood was approximately the same and in the muscles about one half as great. The ratio $\frac{\text{oxybutyric acid}}{\text{total acetone bodies}}$ (β ratio) was low in the blood and high in the tissues. This finding may probably be explained by the fact that conditions in the tissues are favorable for the reduction of acetoacetic acid to oxybutyric acid.²⁸

²⁷ The method employed was shown to give quantitative results when a known amount of acetone was volatilized with the breath of a normal person breathing through the apparatus.

²⁸ For experiments bearing on this point and references to the literature cf. Marriott: this *Journal*, xviii, p. 241, 1914.

TABLE III.

Analysis of blood and organs of a dog fasted and phlorhizinized. Results expressed in terms of acetone per 100 grams of organ.

	ACETONE AND ACETOACETIC ACID	OXYBUTYRIC ACID	TOTAL ACETONE BODIES	β RATIO
Blood.....	7.6	14.0	21.6	65
Liver.....	2.8	19.6	22.4	87
Muscle.....	1.3	9.2	10.5	87

It will be noted that my results are considerably lower than those reported by Sassa and referred to previously in this paper. I am unable to explain this discrepancy.

In the investigation reported in this paper no effort has been made to study any particular type of acidosis but only to ascertain "normal" figures for acetone bodies in the blood and to find what variations from the normal may be expected in a variety of conditions. It is my intention next to apply the methods to the study of those conditions of infancy and childhood that are associated with acidosis.

For placing cases at my disposal and rendering valuable aid during this investigation I am greatly indebted to Dr. George Dock, Dr. G. Canby Robinson and Dr. Borden S. Veeder, and to the members of the house staffs of the Washington University Hospital and of the St. Louis Children's Hospital.

SUMMARY.

1. A revised technique for the determination of acetone bodies in small samples of blood is described.

2. The blood of normal human subjects, and of dogs, pigs and cattle contains less than 1.5 mgm. of acetoacetic acid and less than 4 mgm. of oxybutyric acid per 100 grams of blood, the results being expressed in terms of acetone.

3. In acidosis the acetone bodies in the blood are higher than normal. The highest figure obtained was 28 mgm. of acetoacetic acid and 45 mgm. of oxybutyric acid per 100 grams of blood, expressed as acetone.

THE INFLUENCE OF PROTEIN INTAKE UPON THE FORMATION OF URIC ACID.

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(From the Department of Physiological Chemistry, University of Pennsylvania.)

(Received for publication, June 22, 1914.)

The relation of the common protein metabolism to that of purine is still a matter of controversy. While there is a quite general agreement that the purines of the urine represent the end-product of nucleic catabolism (disregarding the hypoxanthine catabolism of muscle) and in this sense are independent of the common protein catabolism, there is still evidence that in some indirect manner the magnitude of the protein metabolism may exert an influence upon the output of purine. The experiment to be herein reported furnishes an additional illustration of this fact. The subject of the experiment was a healthy man, whose metabolism has been often studied, and whose endogenous purine-nitrogen upon a purine-free diet of moderate nitrogen content was known to run from 0.075 to 0.100 gram. The experiment consisted of a fore-period, in which the subject subsisted upon a practically nitrogen-free diet of purified starch and cane sugar, of a heat value of 2200 calories. Then the man for a period of four days ingested as heavily of white of egg as possible (over 40 grams nitrogen), sugar being added to the diet to make the input of calories equal to that in the first period. The nitrogen, uric acid and creatinine were estimated, the first by the method of Kjeldahl, the others by the colorimetric methods of Folin. The results were as follows:

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DAY	NITROGEN	URIC ACID	CREATININE N
1	7.4	0.28	0.63
2	6.1	0.31	0.62
3	5.4	0.26	0.59
4	4.6	0.30	0.66
5	18.6	0.48	0.68
6	25.3	0.52	0.71
7	28.6	0.78	0.69
8	30.2	0.82	0.68

The output of the uric acid was nearly three times as large upon the heavy purine-free protein diet as upon the protein-free diet. The explanation is not at hand, but two general considerations deserve mention. It is possible, since nucleic acid is synthesized, directly or indirectly, from components of protein, that when the body is flooded with amino-acids, nucleic anabolism and catabolism are exaggerated—an application of the law of mass action. It is possible also to interpret the increased output of uric acid merely as the expression of overwork, the result of the excessive activity of glandular cells in the digestion, assimilation and catabolism of the unusual input of protein. This experiment was undertaken because, in some other experiments we had failed to observe any influence of moderate variations in the protein of the diet upon the uric acid output. Evidently an unusual excess of protein input is required to provoke the phenomenon.

Interesting is the relative constancy of output of creatinine during the experiment, confirming in general the modern idea of the specificity of this metabolism.

ON URICOLYSIS.

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(Received for publication, June 22, 1914.)

The current view of the relations of the catabolism of purine runs to the effect that the end-product of this metabolism is uric acid or allantoin: a small trace of the purine bases accompanies uric acid in man, with a doubtful trace of allantoin of endogenous origin; in many lower animals, as in the dog, allantoin is the chief end product of elimination, with but traces of uric acid and purine bases. The experiments upon which this view is based, the most important of which were carried out by Wiechowski¹ and by Schittenhelm and Seisser² were not regarded by us as entirely conclusive, and an experiment was arranged which we felt could be regarded as free of objection. A female dog was placed upon a diet rich in carbohydrate and low in protein, free of purines in the practical sense. The dog weighed about 10 kilos, and the nitrogenous metabolism under this diet was easily reduced to a gram a day. Under these circumstances, with the parenteral administration of a large dose of allantoin, with direct analysis for urea, uric acid, and allantoin in the urine, the possible conversion into urea could be proved or disproved. The allantoin was prepared from uric acid by oxidation, and purified by repeated recrystallization until the correct figure for nitrogen was attained. It was administered as the sodium salt, dissolved in 200 cc. of Ringer's solution. 5.634 grams of allantoin were thus introduced corresponding to 2 grams of nitrogen, which figure contrasts strongly with that for the daily output of urea nitrogen. The allantoin was injected under ether anesthesia into the femoral vein. The

¹ Wiechowski: *Arch. f. exp. Path. u. Pharm.*, lx, p. 295, 1909.

² Schittenhelm and Seisser: *Zeitschr. f. exp. Path. u. Therap.*, vii, p. 116, 1909.

urine was collected in twenty-four hour periods. Allantoin was estimated by the method of Wiechowski, and on the experimental day the value obtained by this method was checked with values obtained by the method lately suggested by Plimmer and Skelton.³ These investigators showed that the difference between the figures obtained for urea by the Folin method and by the urease method represents allantoin nitrogen. On the experimental day the figure in the column of the table marked "actual urea nitrogen" represents that obtained by the urease method. On the other days the urea was determined by Benedict's method and as Benedict's method was found invariably to account for 75 per cent of the allantoin nitrogen present, correction was made for this amount in the column marked "actual urea nitrogen." Uric acid was estimated colorimetrically according to the method of Folin and Denis.

DAY	WEIGHT	TOTAL N	UREA N BENEDICT "	UREA N " ACTUAL "	ALLANTOIN N	URIC ACID	REMARKS
	kgms.	gms.	gms.	gms.	gms.	gm.	
1	9.78	1.09	0.83	0.70	0.18	0.014	
2	9.79	1.04	0.80	0.70	0.14	0.010	
3	9.93	1.26	0.94	0.84	0.13	0.012	
4	9.97	2.96	2.09	0.69	1.84	0.031	Allantoin = 2 grams N injected intravenously
5	9.79	1.23	0.99	0.84	0.20	0.016	
6	9.91	1.04	0.82	0.71	0.14	0.015	
7	9.74	0.93	0.74	0.63	0.14		

These figures would seem to demonstrate directly the correctness of the view that in the dog allantoin is an end product and is not converted into urea; or in other words using uric acid and allantoin as synonymous terms, there is no uricolysis in the dog. The figure for urea was not changed by the administration of the huge dose of allantoin. The allantoin was nearly all recovered in the urine voided within twenty-four hours after the administration of the allantoin. This elimination was so prompt, complete, and devoid of any indication of oxidation or other alteration of the allantoin as to warrant the conclusion that allantoin of exogenous

³ Plimmer and Skelton: *Biochem. Journ.*, viii, p. 70, 1914.

origin behaves in the dog as a foreign body. Certainly there is nothing in these figures to suggest that allantoin is an intermediary body in metabolism. The urine of the dog contains little uric acid, and the estimation of this small amount is not entirely satisfactory by the Folin method on account of the high concentration of coloring bodies in the residue after the ether-methyl alcohol extraction of the dog's urine. Nevertheless, the figures may be regarded as approximately correct. The figure for uric acid rose on the day of the administration of allantoin, indicating probably the reversion of the reaction, $\text{uric acid} \rightleftharpoons \text{allantoin}$, a state of affairs that should occasion no surprise.

If allantoin in the dog is not converted into urea, then it would seem very certain that in man uric acid is not converted into urea.

OBSERVATIONS ON CREATINE AND CREATININE.

BY PHILIP A. SHAFFER.

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New York, and the Laboratory of Biological Chemistry,
Washington University, St. Louis.)

The recent appearance of a series of papers by Folin and his collaborators¹ describing important modifications in the technique of his method for the determination of creatinine and creatine as applied to blood, milk and tissues, and giving extensive data as to the amount of these substances present in blood and muscles, as well as the interesting papers by Myers and Fine² and by Baumann,³ prompts the publication of a number of experiments and analyses on the same subject and obtained with somewhat similar technique by Doctor R. A. Hatcher and myself and by Mr. E. A. Reinoso and myself between 1907 and 1910.⁴ The publication of our results was postponed because of the expectation of resuming and extending the work, which up to the present has not been undertaken.

The data here reported represent an uncompleted part of a study of creatine and creatinine planned about eight years ago. Our work was directed along two lines: the factors which influence the excretion of creatinine and creatine, data concerning which were published in 1908; and the search in the organs of the body for the place of origin of the creatinine excreted in the urine. Concerning the excretion of creatinine it was shown⁵ that the amount excreted by a given subject on a creatinine-free diet is remarkably constant

¹ This *Journal*, xvii, 1914.

² Myers and Fine: *ibid.*, xiv, xv, xvi and xvii.

³ Baumann: *ibid.*, xvii.

⁴ The work was done in the Laboratory of Chemical Pathology, Cornell Medical School, New York.

⁵ Shaffer: *Amer. Journ. of Physiol.*, xxiii, p. 1, 1908; also *Arch. of Int. Med.*, iv, p. 588, 1909.

not only from day to day (Folin) but from hour to hour; that the amount excreted is independent of the degree of muscular activity; that the relative amount expressed as milligrams of creatinine-nitrogen per kilo of body weight (creatinine-coefficient), is directly parallel to the muscular development and power of the subject; and that therefore creatinine is probably to be considered as "derived from some special process of normal catabolism taking place largely if not wholly, in the muscles."

Endogenous creatine on the other hand, as is now well known, is not excreted normally, but does appear in the urine during acute fevers (on the usual "light hospital diets)," in cachexias and wasting diseases such as the acute stages of exophthalmic goitre and tumor cachexia, and in especially large quantities during the first week post partum.⁶ It is scarcely to be doubted that in all of these conditions muscle tissue is being broken down and that the creatine excreted is merely creatine thus freed from the muscle cells.

The difference in behavior of endogenous creatine and creatinine served to emphasize the difficulty of assuming without further evidence that the normal urine creatinine is directly derived from muscle creatine although as above mentioned, the facts clearly pointed in that direction. It was thought that a knowledge of the relative amounts of creatinine and creatine in blood, muscle and other tissues might throw further light on the question.

Work on this latter point involved the detection and determination of exceedingly small quantities of creatinine in the presence of relatively large amounts of creatine and other disturbing factors, and necessitated at the start an examination of modifications in technique which might suit the conditions. A few of the results of these experiments were reported in abstract in 1909,⁷ but were not published in full because of their rather fragmentary character. In view of the revival of interest and activity in this direction and because of the similarity of our technique to that recently described by Folin, it appears desirable to publish some of the material in its present form in order that it may be available to other workers.

⁶ Shaffer: *loc. cit.*

⁷ Shaffer and Reinoso: Note of the Determination of Kreatinin, *Proc. Am. Soc. of Biol. Chem.*; this *Journal*, vii, p. xiii, 1910; Shaffer and Reinoso: Do Muscle and Blood Serum Contain Kreatinin? *ibid.*, vii, p. xxx, 1910.

The determination of creatinine especially in very dilute solutions.

The conditions originally prescribed by Folin for the determination of creatinine in urine, and which have been very extensively adopted, require that an amount of urine be used which contains between 7 and 15 mgm. of creatinine, to which is added 15 cc. of saturated (1.2 per cent) picric acid solution and 5 cc. of 10 per cent sodium hydroxide. After standing five minutes the mixture is diluted to 500 cc. and the color compared with that of $\frac{N}{2}$ potassium bichromate. Seven to 10 mgm. of creatinine are usually contained in 20 cc. or less of urine and in such concentration the results are accurate; but in greater dilutions such as are encountered in the urine of diabetics or other subjects of polyuria, the urine of infants, as well as the urine of many subjects whose creatinine excretion is very small, and especially in attempts to apply the method to muscle or other tissue extracts, the results are too low depending upon the extent of the dilution. When the volume of urine or creatinine solution is more than about 35 cc., the results become progressively lower until with a volume of 200 cc. they may indicate only about 25 per cent of the amount actually present. This error limits the use of the method, unless further modified, to solutions which contain 20 mgm. or more of creatinine in 100 cc. But with slight modifications the method becomes applicable to solutions containing only about 1 mgm. or less in 100 cc., and may be used for the determination of preformed creatinine in blood or muscle extracts.

The speed of the formation of the red reduction compound of picric acid by the reaction with creatinine is determined by the concentration of picric acid, alkali and creatinine, and maybe hastened and made more complete and permanent by increasing the amounts of picric acid and alkali. A definite proportion must be maintained between the picric acid and alkali, but when this is done, increasing the amounts is without effect upon the result. With 10 cc. of creatinine solution (7 to 10 mgm.) and 440 cc. of picric acid, the reading is the same as when carried out according to Folin's directions.

As a result of several hundreds of determinations under different conditions we concluded that the optimum conditions are: an

amount of saturated picric acid solution equal to the volume of creatinine solution and one-tenth this volume of 10 per cent sodium hydrate. After standing six to ten minutes, the liquid is diluted to a definite volume depending upon the intensity of the color developed. With very dilute solutions one may add solid picric acid equivalent to half saturation (0.6 per cent) and when dissolved, one-twentieth the volume of sodium hydroxide. Provided the creatinine solution itself has not sufficient color to interfere, the results are as accurate as according to Folin's original directions

TABLE I.

The amount of creatinine was constant for each series of determinations. After standing six minutes the liquid was diluted to 500 cc. with filtered tap water at room temperature and the solution compared with $\frac{N}{2}$ potassium bichromate in a Duboseq colorimeter set at 8 mm.

SERIES	NO.	VOL. OF CREATININE SOLUTION	SATURATED PICRIC ACID	10 PER CENT SODIUM HYDROXIDE	AVERAGE READING	CREATININE FOUND	CREATININE PRESENT*
		cc.	cc.	cc.	mm.	mgm.	mgm.
I	1	10	15	5.0	11.05	7.32	7.32
	2	10	15	20.0	13.20	6.15	7.32
	3	10	30	5.0	11.05	7.32	7.32
	4	50	15	5.0	12.00	6.75	7.32
	5	50	30	5.0	11.70	6.95	7.32
	6	60	60	10.0	11.05	7.32	7.32
	7	100	15	5.0	13.20	6.15	7.32
	8	100	30	5.0	11.80	6.85	7.32
II	1	10	15	5.0	8.00	10.10	10.10
	2	10	30	1.0	16.20	5.00	10.10
	3	10	30	2.0	8.20	9.90	10.10
	4	10	30	3.0	8.10	10.00	10.10
	5	10	30	4.0	8.10	10.00	10.10
	6	10	30	5.0	8.00	10.10	10.10
	7	200	30	5.0	26.00	3.10	10.10
	8	400	30	15.0	19.20	4.20	10.10
	9	110	120	8.5	8.00	10.10	10.10
	10	110	120	13.0	7.95	10.20	10.10
	11	210	200	17.0	8.00	10.10	10.10
	12	210	240	17.0	8.00	10.10	10.10
	13	250	200	20.0	8.10	10.00	10.10
	14	250	200	20.0	8.15	9.95	10.10
	15	10	360	25.0	8.00	10.10	10.10
	16	10	240	17.0	7.95	10.20	10.10

* Result of determination according to Folin's directions.

and will probably be found as accurate as those obtained according to Folin's very recent description. When only small amounts of solution are available, it is of course equally convenient and accurate to dilute to a correspondingly smaller volume. For instance 20 cc. of creatinine solution (containing 0.8 mgm.) plus 20 cc. of picric acid plus 2 cc. of alkali diluted to 50 cc., or 45 cc. (containing 0.8 mgm.) plus 0.24 gram of picric acid plus 2 cc. of alkali and diluted to 50 cc. gives the same reading as 200 cc. of solution containing 8 mgm. plus 200 cc. of picric acid plus 20 cc. of alkali diluted to 500 cc., and the same as 10 cc. of creatinine solution containing 8 mgm. carried out as usual. The results in Table I illustrate these factors, the effect of dilution, amounts of alkali and of picric acid.

The effect of protein upon the determination of creatinine.

Neither albumin or globulin, proteoses or peptones in the amounts which may be present in tissues extracts have any considerable effect upon the intensity of color developed within ten or fifteen minutes with picric acid and alkali, and it is therefore not necessary to remove protein from solutions in which it is desired to determine creatinine. In the presence of proteins larger amounts of picric acid and alkali should be used. The results in Table II serve to illustrate:

TABLE II.

CREATININE SOLUTION	WATER ADDED	PROTEIN ADDED	PICRIC ACID	10 PER CENT NaOH	CREATININE FOUND
cc.	cc.		cc.	cc.	mgm.
10	100	0	100	10	10.0
10	140	20 grams egg white	150	15	10.05
10	20	2 grams egg white	30	5	10.10
10	50	25 cc. blood serum	100	10	10.20
10	0	5 cc.	30	5	10.05
10	140	50 cc.	200	20	10.77 (equivalent to 1.5 mgm. creati- nine in 100 cc. serum)
10	90	1 gram Witte's peptone	100	10	9.74
10	90	5 grams Witte's pep- tone	100	10	8.4
10	10	0.5 gram Witte's pep- tone	200	17	10.05

Determination of creatinine in blood and tissue extracts.

With blood, hemolyzed serum or blood-tinged extracts, however, the color of the hemoglobin prevents the use of any colorimetric method unless the hemoglobin is removed. In our work we used the process of precipitation with picric acid which has very recently been used by Folin. This method of precipitation is an important addition to such technique because it yields clear yellow filtrates which are void of all interfering color and is therefore very serviceable in the determination of very small amounts of creatinine in blood-colored liquids. It is not applicable for use with solutions which may contain more than about 20 mgm. of creatinine in 100 cc. because a part of the creatinine may be precipitated with or absorbed by the protein. A series of our experiments showed that on adding a known amount of creatinine to blood and muscle there is practically no loss if the amount present is less than about 10 mgm. in 100 cc. of final extract.

The procedure which we used for the determination of creatinine in blood serum, milk, amniotic fluid, "meat extracts," and in most cases with muscle and other tissue extracts, was as follows: With muscle and other tissues, 50 to 100 gram samples of the hashed material were extracted six times with successive portions of about one and one-half volumes of cold water.⁸ During each extraction the mixture was stirred or shaken for about thirty minutes and the liquid strained through cheese cloth without loss. The combined extracts were diluted to definite volume and filtered.

To such tissue extracts and to blood serum and the other liquids was added an equal volume of saturated aqueous solution of picric acid or picric acid in substance. After thorough shaking, stirring or rubbing, the mixture was filtered and a measured portion of the clear filtrate was used for the creatinine determination. The depth of color developed on the addition of alkali was, in most cases, too weak for accurate comparison with $\frac{N}{2}$ potassium bichromate, and in order to obtain a suitable color, a known amount of pure creatinine was added to another portion of the picric acid filtrate. The 10 per cent sodium hydroxide was next added⁹ and the mixture, contained in a

⁸ In some cases instead of repeated extraction portions of muscle were thoroughly stirred or shaken for several hours with one or two volumes of water and the liquid strained, centrifugated and filtered. The total volume of extract was assumed to be 170 cc. or 270 cc. respectively per 100 gram, muscle, and the results calculated accordingly.

⁹ The amount added must be approximately equal to one-tenth the total volume of the saturated picric acid.

volumetric flask, was immediately diluted to the mark with saturated picric acid solution and well mixed.¹⁰ After standing six minutes the liquid was filtered and read against $\frac{N}{2}$ potassium set at 8 mm. in a Duboseq colorimeter. The amount of creatinine added was in each instance determined simultaneously under the same conditions as regards volume of solution, total dilution and amounts of picric acid and alkali, and the amount of creatinine in the serum or extract calculated from the difference between the results so obtained. Duplicate analyses as a rule agreed within a few hundredths of a milligram for a total volume of 50 cc.

The following details illustrate the procedure: *Blood serum.* 100 cc. of diluted dog serum (= 77 cc. of serum) + 100 cc. of picric acid solution and a small amount of picric acid crystals. 25 cc. of the clear filtrate (= 9.6 cc. of serum) + 1 cc. of creatinine solution (0.684 mgm. creatine) + 4 cc. of 10 per cent NaOH, diluted to 50 cc. with picric acid solution, read after six minutes.

	10.1 mm.	=	0.80 mg. creatinine
Duplicate	10.0		0.81 mg. creatinine
			<hr/>
	Average		0.805 mg. creatinine
Less creatinine added			0.684 mg. creatinine
			<hr/>

0.121 mg. creatinine in 9.6 cc. of serum or 1.27 mgm. in 100 cc.

Separate determinations with the same serum gave 1.12, 1.04 and 1.64 mgm. per 100 cc.

Blank on creatinine solution: 25 cc. of picric acid solution + 1 cc. of creatinine solution + 4 cc. of NaOH, diluted to 50 cc. with picric acid solution. Average readings = 11.85 mm. = 0.684 mgm. creatinine.

Muscle. 185 grams hashed human muscle extracted repeatedly with cold water and the combined extracts diluted to 1000 cc. 250 cc. were saturated by shaking with 3 grams of picric acid. 100 cc. of filtrate + 2 cc. of creatinine solution (1.62 mgm.) + 10 cc. of 10 per cent sodium hydroxide diluted with water to 200 cc.

$$\text{Average reading} = 9.1 \text{ mm.} = \frac{8.1}{9.1} \times \frac{200}{500} = 3.56 \text{ mgm.}$$

3.56 - 1.62 = 1.94 mgm. creatinine or 19.4 mgm. in 1000 cc. extract from 185 gram muscle or 10.5 mgm. in 100 gram muscle

Duplicate determinations gave 11.2 11.2 and 11.7 mgm.

Alcohol extraction.

During a part of our work the muscle tissue was repeatedly extracted with water and alcohol, first twice with two volumes of water, followed by two extractions with two volumes of 95 per cent alcohol. The combined extracts

¹⁰ The final volume should not be greater than twice the amount of the tissue filtrate used; see the examples given above.

were filtered from the protein precipitated by the alcohol and the whole filtrate evaporated *in vacuo* below 30°C. The residue was dissolved in water, filtered and the whole of the solutions representing from 50 to 100 grams of muscle were used for creatinine determination by the addition of an equal volume of picric acid solution and one-tenth the amount of 10 per cent sodium hydroxide. The following are some of the results obtained in this way. Control determinations in which pure creatine solutions were carried through the evaporation showed that under the conditions there is no conversion of creatine into creatinine. But on the other hand, the results are somewhat low as compared with those obtained by water extraction, probably because of incomplete extraction or absorption by the alcohol precipitate. Creatine determinations (creatine and creatinine) in the alcohol extract gave results only about half of those obtained by water extraction.¹¹

TABLE III.
Alcohol extraction.

	MGM. PRE-FORMED CREATININE IN 100 GRAMS	MGM. TOTAL CREATININE IN 100 GRAMS
Fresh cats' skeletal muscle.....	1.90	
Fresh dogs' skeletal muscle.....	3.15	
Fresh dogs' skeletal muscle.....	6.00	195.0
Fresh dogs' skeletal muscle.....	5.60	
Fresh dogs' skeletal muscle.....	4.20	
Fresh dogs' kidney.....	3.30	9.0

The conversion of creatine into creatinine.

The results in the following table indicate the effect of concentration of acid and time of heating (water bath) on the conversion of creatine into creatinine. To 10 cc. of creatine solution, containing about 8 to 12 mgm., was added enough hydrochloric acid to give the concentration stated and water added to a volume of 15 cc. After heating, the acid was neutralized with an equivalent amount of sodium hydroxide, 15 cc. of picric acid and 5 cc. of 10 percent sodium hydroxide added. After six minutes the mixture was diluted to 500 cc. and read against $\frac{N}{2}$ potassium bichromate set at 8 mm.

These and other experiments show that final concentrations of 1N to 3N hydrochloric or sulphuric acids accomplish the complete conversion of small amounts (up to 50 mgm.) of creatine into creatinine without loss by decomposition, within two or three hours at the temperature of a boiling water bath. The solutions were heated in small Erlenmeyer flasks, covered with watch glasses to retard evaporation. In view of the satisfactory results by this method we did not use the autoclave.

¹¹ The values published by Chisolm (*Biochem. Journ.*, vi, p. 243, 1912) for the creatine content of normal human muscle are probably too low. They were obtained by Mellanby's alcohol extraction process.

TABLE IV.

Pure creatine solutions.

SOLUTION I			SOLUTION II		
Time of heating	Final concentration HCl	Creatinine found	Time of heating	Final concentration acid HCl	Average creatinine found
<i>hrs.</i>		<i>per cent</i>	<i>hrs.</i>		<i>mgm.</i>
3	0.2 N	4.3	1	1.0 N	7.90
3	0.3 N	4.6	2	1.0 N	8.10
3	0.5 N	5.30	3	1.0 N	8.10
3	0.6 N	5.27	1	2.7 N	8.03
3	0.8 N	5.30	2	2.7 N	8.12
3	1.1 N	5.70	3	2.7 N	8.12
3	1.4 N	5.70			
3	1.6 N	5.70			
3	2.2 N	5.70			
3	2.7 N	5.70			

Determination of total creatinine in muscle.

For most of our determinations we used the combined water extracts of muscle prepared by six repeated extractions. The following results of the determinations in the separate extracts indicate that the creatine was fairly completely extracted, though as Folin points out, such results doubtless represent minimum rather than maximum values.

	MGM. TOTAL CREATININE PER 100 GRAMS MUSCLE	PER CENT OF TOTAL
First extract	227	70.7
Second extract	81	25.2
Third extract	13	4.1
Total.....	321	
First extract	184	65.6
Second extract	61	21.8
Third extract	21	7.5
Fourth extract	12	4.3
Fifth extract	1	0.4
Sixth extract	1	0.4
Total.....	280	

During the latter part of our work we heated a weighed portion (10-20 grams) of the hashed muscle with 10 volumes of $\frac{N}{2}$ HCl or H_2SO_4 on a water

bath for five hours, during which time the tissue largely dissolved.¹² The acid was then neutralized, the solution diluted to definite volume and filtered. To 30-cc. portions of the filtrate was then added acid to make 2N and the mixture heated two hours to assure the complete conversion of the creatine. The determination was then carried out as above described. With this method the results were somewhat higher than those obtained by extraction. Thus 100 grams of human muscle contained with five extractions with cold water 362 mgm. creatine, by direct heating with 2 per cent HCl, 399 mgm. creatine. Such differences were several times obtained and probably represent the extent of the loss by water-extraction methods.

The presence of protein during the heating with acid does not interfere with the conversion of creatine nor with the later color determination, as we assured ourselves by many experiments. For instance in a muscle extract containing extracted protein was found 386 mgm. total creatinine for 100 grams of muscle. In the same extract the protein was removed by heat coagulation before the conversion of creatine and the result then obtained was 390 mgm. This agrees with the findings by Baumann¹³ and Folin.¹⁴

With the methods above described we made a considerable number of determinations of preformed creatinine and total creatinine and creatine in skeletal and heart muscle of several species and in blood and other fluids. The more reliable of our results are given in the following tables (V, VI and VII).

TABLE V.

TISSUE	PREFORMED CREATININE	TOTAL CREATINE	REMARKS
Cat skeletal muscle...	4.0	372	Fresh muscle from thigh.
Sheep skeletal muscle.		324	Some days old—bought in market.
Pig skeletal muscle...		408	Some days old—bought in market.
Beef skeletal muscle...		365	Some days old—bought in market.
Beef skeletal muscle...	10.3	450	Some days old—bought in market.
Beef skeletal muscle...	{ 12.7a 12.4b		a. Folin's new method. b. Same extraction process as used in other analyses. Recent analyses on same sample.
Human amniotic fluid...	2.0	3.5	

¹² The plan of hydrolyzing the whole tissue largely adopted by Baumann and Folin appears to have been first used successfully by Pekelharing and v. Hoogenhuyze: *Zeitschr. f. physiol. Chem.*, lxiiv, p. 264, 1910.

¹³ This *Journal*, xvii, p. 1914.

¹⁴ This *Journal*, xvii, p. 1914.

TABLE VI.

Creatine and Creatinine in fresh dog blood and tissues.

The analyses were made within a few hours after death of the animals.
Results expressed in mgms. per 100 grams of tissue.

TISSUE	PREFORMED CREATININE	TOTAL CREATINE	REMARKS
Dogs' blood.....	0.8	7.4*	Blood of normal dogs, drawn from carotid. Analysis within a few hours.
Dogs' blood.....	1.4		
Dogs' blood.....	1.0		
Dogs' blood.....	1.6		
Dogs' blood.....	1.3		
Dogs' blood.....	1.1		
Dogs' blood.....	1.0		
Dogs' blood.....	1.6		
Dogs' blood.....	8.5	371 376 425 270 382 472†	Blood drawn 24 hours after ligation of both renal arteries.
Dogs' skeletal muscle..			Fasting, extreme emaciation.
Dogs' skeletal muscle..	9.7		
Dogs' skeletal muscle..			
Dogs' skeletal muscle..			
Dogs' skeletal muscle..			
Dogs' skeletal muscle..	5.0		
Dogs' skeletal muscle..	11.5		
Dogs' skeletal muscle..	15.6		
Dogs' skeletal muscle..	13.0		
Dogs' skeletal muscle.,	10.0		
Dogs' heart muscle....		225	
Dogs' heart muscle....		197	
Dogs' liver.....	1.2	15	
Dogs' kidney.....		29	

* Protein removed by heat coagulation and evaporation of filtrate.

† Tissue dissolved by HCl on water bath.

TABLE VII.

Creatinine and creatine in human muscle.

Results are expressed in mgm. per 100 grams muscle.

SUBJECT	PREFORMED CREATININE	TOTAL CREATINE	TOTAL NITROGEN IN WATER EXTRACT
I. Well developed young laborer. Sudden death by accident.....	11.5	410	708
II. Suicide by morphine.....	10.0	430	
III. Well developed man. Sudden acute nephritis, death in coma.....	10.0	393	
IV. Diabetes, death in coma.....	7.5	391	950
V. Diabetes.....	6.3	370	
		399*	
VI. Fatal acute exophthalmic goitre.....	4.3	342	

* Tissue dissolved by acid on water bath.

DISCUSSION.

From these results it appears that dog blood serum normally contains about 1 or 2 mgm. of creatinine in each 100 cc., amounts similar to those recently reported by Folin and Denis¹⁵ for man and other species. Skeletal muscle tissue of a number of species, after the lapse of about six hours (the time required in our determinations) or longer after death, is found to contain from 5 to 15 mgm. in each 100 grams. In several instances much lower results were obtained with muscle tissue and it is quite possible that the larger amounts usually found, in part represent creatinine formed in the muscles after the stoppage of the circulation. This important point requires further investigation and in such work the recent Folin plan of adding picric acid at once to the hashed freshly removed muscle probably should be used. Whatever the result of such investigation, the important point remains that *muscle tissue within a few hours after death contains from five to ten times as much creatinine as does the blood which passes through it, and much more than the liver, to which has been ascribed the formation of creatinine* (Mellanby). This fact is in our opinion very strong evidence that the muscles are the tissues in which creatinine is formed. The analyses here published were used as the basis for this argument by Reinoso

¹⁵ Folin and Denis: this *Journal*, xvii, p. 487, 1914.

and the writer in 1909.¹⁶ Evidence of the same character has recently been brought forward by Folin and Denis,¹⁷ who found from 2.3 to 8 mgm. of creatinine per 100 grams of skeletal muscle with five cats, as compared with 1 to 2 mgm. in blood. Myers and Fine state¹⁸ that they find about 3 to 10 mgm. of creatinine per 100 grams of muscle, but they give few details. Thus the results so far obtained for the amounts of creatinine in muscle and blood are in substantial agreement and are of the order which might have been expected from the standpoint of the amount of creatinine excreted.

It is clear that the reason creatinine has not been detected in muscle and blood by other investigators¹⁹ is that too large amounts have been looked for. If it be assumed that the urine creatinine is formed in the muscles, we should expect on the basis of the muscles amounting to about half the body weight that 50 mgm. of creatinine is formed by 1 kilo of muscle per day or about 0.2 mgm. for 100 grams of muscle per hour. In view of the blood circulation and the known speed and ease of excretion of creatinine it would be distinctly surprising to find any considerable amounts of this substance anywhere in the body. The amounts²⁰ actually found in muscles, small as they are, are nevertheless many times greater than found elsewhere in the body, and are approximately equivalent to the amount excreted in twenty-four hours. If it can be satisfactorily shown, as recently announced by Myers and Fine,²¹ that muscle on autolysis forms creatinine at the expense of creatine, the origin of the former would appear to be established.

¹⁶ Shaffer and Reinoso: *Proc. Amer. Soc. of Biol. Chem.*, 1909: this *Journal*, vii, p. xxx, 1910.

¹⁷ Folin and Denis: this *Journal*, xvii, p. 501, 1914.

¹⁸ Myers and Fine: *Proc. Soc. Exp. Biol and Med.*, xi, p. 15, 1913; this *Journal*, xv, p. 304, 1913, xvii, p. 68, 1914.

¹⁹ Grindley and Woods: this *Journal*, ii, p. 309, 1907; Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908; Chisolm: *Biochem. Journ.*, vi, p. 243, 1912.

²⁰ The above calculation as to the rate of creatinine formation makes it probable that the amounts found in the case of analyses made within a few hours represent very nearly the amounts actually present in the muscle during life, for a continuation of the assumed normal rate of creatinine formation during about four hours (in the dog muscle analyses) would increase the amount of creatinine present by at most 1 mgm. per 100 grams of muscle.

²¹ Myers and Fine: this *Journal*, xv, p. 304, 1913.

The autolysis experiments²² so far reported on this point are far from conclusive because of unsatisfactory technique for the determination of such small amounts of creatinine and the probability of the conversion of creatine into creatinine during the analytical procedure. The whole subject of the enzymes which have been claimed to participate in the transformation of creatine and creatinine within the body is urgently in need of revision by improved technique, and we hope in the near future to undertake work along this line.

In view of the large number of observations which have now been reported on the creatine content of muscles of different species, the results on this point here presented are too few for generalization. Our values vary considerably more than do those of Myers and Fine²³ though in general the averages are not greatly different. Both our results and those of Myers and Fine are somewhat lower than those of Folin and Buckman,²⁴ perhaps because of somewhat incomplete extraction from the tissues. Although from the very recent determinations by Baumann,²⁵ comparing his process of hydrolyzing the whole tissue with the water extraction according to Myers and Fine, it would appear that both methods give practically the same results. Our results on dogs' skeletal muscle, taken from the thigh in each case, vary from 270 mgm. to 472 mgm. per 100 grams of muscle. Excluding the one exceptionally low value (270 mgm.) the average is 405 mgm. as compared with the Myers and Fine average of 367 mgm. We unfortunately did not determine the water or nitrogen content of the tissues and it is possible that the differences noted with different animals are due to different degrees of hydration.

With six analyses of human muscle²⁶ we made simultaneous determinations of preformed creatinine and total creatinine and

²² Gottlieb and Stangassinger: *Zeitschr. f. physiol. Chem.*, lii, p. 1, 1907. Iv, p. 295, 1908.

²³ Myers and Fine: this *Journal*, xiv, p. 9, 1913, which contains a summary of other analyses.

²⁴ Folin and Buckman: this *Journal*, xvii, p. 483, 1914.

²⁵ Baumann: this *Journal*, xvii, p. 17, 1914.

²⁶ The muscles (pectoralis major and rectus abdominis) were removed at autopsy, kept on ice and analyzed from twenty-four to thirty-six hours after death. I am indebted to Doctors James Ewing, William Elser and Douglas Symmers for this material.

creatine, and the relationship between the two is very suggestive. The amount of creatine found varies from 342 to 430 mgm. as compared with results of Myers and Fine²⁷ of 396 (peritonitis), 391 (sarcoma of leg), 321 (infant, cerebrospinal meningitis), and 311 (oedema). Chisolm²⁸ reports values of 257, 290, 271, 251, 280 and 263 mgm. per 100 grams of skeletal muscle of six healthy adults. The reason for the lower results of Chisolm is probably due to his use of the alcohol extraction method, and like Myers and Fine, I hesitate to accept them as representing normal human muscle. The important point brought out by Chisolm is nevertheless I believe probably correct, namely that the muscles of subjects of chronic diseases who have lost much body weight contain relatively less creatine than do the muscles of more healthy subjects. The same principle is suggested by the results above cited from Myers and Fine, and is supported by our analyses.

Three of our subjects may be classed as having a normal muscular development and very probably a normal creatinine excretion. In these cases the creatine content was 430, 410 and 393 mgm. Of the remaining subjects two were diabetics and one a fatal case of exophthalmic goitre, in both of which conditions in the advanced stages it is known that the "muscular efficiency" and also the creatinine excretion are markedly below the normal. In these cases the muscle creatine amounted to 391, 370 and 342 mgm., respectively, or somewhat lower than with the three more normal subjects. The preformed creatinine shows a similar relationship, being 11.5, 10 and 10 mgm. per 100 grams of muscle in the well developed individuals, and 7.5, 6.3 and 4.3 mgm. respectively in the subjects of diabetes and Grave's disease. The low values from the subject of Grave's disease are further emphasized by the relatively high protein content of the water extract, 950 mgm. nitrogen as compared with 708 mgm. in one of the normal subjects, the only other instance in which that determination was made.

It is freely acknowledged that these observations are too few in number to justify any conclusion, but it would appear to be more than a coincidence that the muscles of the subjects which quite certainly had low creatinine coefficients contained less creatine and less creatinine than was found in the case of the supposedly more

²⁷ Myers and Fine: *this Journal*, xiv, p. 17, 1913.

²⁸ Chisolm: *Biochem. Journ.*, vi, p. 243, 1912.

nearly normal individuals. If it should be shown as stated by Myers and Fine that the amount of creatinine in muscle increases after removal from the animal, the relative creatinine values here given of course lose a portion of their significance for the reason that varying periods, twenty-four to thirty-six hours, elapsed before analysis; but several preliminary experiments which we carried out on this point did not indicate this to be the case for such periods of time. This relationship if confirmed by future work, will be additional strong evidence in favor of the hypothesis long held but still without wholly sufficient proof, that the creatinine of urine has its origin in muscle creatine.

The relationship between the amount of muscle creatine and the creatinine excretion has been well emphasized by Myers and Fine²⁹ for three different species, rabbit, man and dog. Chisolm's results point to the same conclusion in respect to normal and pathological human subjects, though he made no interpretation of his muscle creatine values in relation to creatinine excretion. Our results appear to confirm the above relationship and suggest further that the amount of preformed creatinine present in the muscles of an animal perhaps is in proportion to the amount excreted; and our results together with those of Chisolm indicate that these factors apply not only to different species, but to the different amounts of creatinine excreted (creatinine coefficients) by members of a single species. But the questions cannot be considered settled; a larger number of controlled analyses by satisfactory methods are required. In the meanwhile the facts long known in regard to the relation between the mass and degree of development of muscle tissue of an individual and the amount of creatinine excreted, together with the larger amount of preformed creatinine found in muscle as compared with the amount in blood and other tissues, and the apparent relationship between the relative amounts of muscle creatine and creatinine and the creatinine excretion (creatinine coefficient), point definitely to the conclusion that creatinine of urine is formed in the muscle from creatine (or creatine-containing complex, Folin-Denis) and in proportion to the intensity of the *normal* endogenous reactions of life.

²⁹ Myers and Fine: this *Journal*, xiv, p. 18, 1913.

A SIMPLIFICATION OF THE DETERMINATION OF TOTAL NITROGEN BY COLORIMETRY.

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The colorimetric method for the determination of the total nitrogen in urines was introduced by Folin and Farmer¹ in 1912. Its rapidity makes it considerably superior to previous methods in medical work, but it requires apparatus and laboratory facilities that render it unlikely to spread far outside of the larger institutions.

In essence, their method is to oxidize a minute portion of the urine according to the Kjeldahl-Gunning method, then make alkaline and aspirate the ammonia over into a little acid, after which it may be estimated colorimetrically by a modification of Nessler's ammonia method. The most troublesome part of this procedure is the quantitative aspiration of the ammonia out of the original mixture into a new solution—an operation which Folin regretted to employ, but did not find it possible to avoid because when the ammonia was nesslerized in the oxidation mixture, the colored solution was very prone to be turbid and unfit for a colorimetric determination.

More than a year ago the writer noted that if sulphuric acid and potassium sulphate were present in only very small quantities they did not interfere seriously with the Nessler color reaction. Thus the hope was aroused that by devising suitable conditions the entire second step of Folin's method—the aspiration—might be omitted, and the method would then consist simply in oxidizing the urine in a very small quantity of acid mixture, nesslerizing the product in a volumetric flask and estimating the color by the use of any good colorimeter.

¹ This *Journal*, xi, p. 493, 1912.

I. Modifications of the Kjeldahl oxidation.

A. MICROCHEMICAL METHOD: Sufficient urine to contain about 0.5 mgm. of nitrogen is oxidized in a minimal quantity of acid. The key to a successful oxidation is the use of a suitable flask so made as to conserve the acid and avoid loss of nitrogen by spattering. The best form seems to be a flask with a bluntly pointed bottom

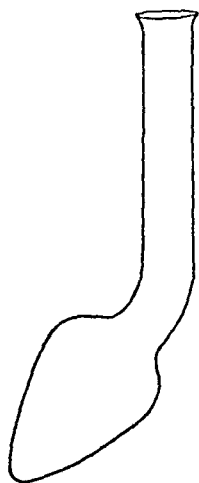


FIG. 1. Micro-oxidation flask
($\times \frac{1}{2}$).

(inverted pear shaped) and with a bent neck, as shown in the text figure. The capacity of the bulb is about 15 cc. In use, the bulb is set at a slant, and the mouth is directed upward. Heat can be applied at the tip of the bulb without overheating the other portions, and if the slant is set right, the acid that distills up can flow back continually into the tip of the bulb.

Under these conditions, if due precautions are taken to prevent "bumping" and spattering, the requisite amount of urine can be completely and satisfactorily oxidized in about ten minutes, in the presence of 0.1 cc. or even less of sulphuric acid, 0.05 gram of potassium sulphate, and a trace of mercuric salts.

A solution containing the oxidizing mixture can be made up conveniently as follows:

Ammonia-free water, about.....	125 cc.
Sulphuric acid.....	40 cc.
Saturated solution of mercuric chloride.....	5 cc.
Potassium sulphate.....	20 grams
Ammonia-free water, enough more to complete a total volume of 200 cc.	

Procedure. By means of the above acid mixture dilute a sample of urine to an exact multiple of its original volume (about 4-10 times), so that 0.5 cc. of the dilution will contain between 0.4 mgm. and 0.7 mgm. nitrogen.

With an Ostwald pipette introduce 0.5 cc. into the bulb of the micro-oxidizing flask. Add a small spherical glass bead or a scrap of platinum to aid the boiling. (Best of all is a piece of platinum wire, 4-5 mm. long, bent into a tight spiral. Quartz sand can be

used, but it tends to be thrown up and lodge on the sides of the dish.)

Agitate continually while boiling off the water over a micro-burner (about one minute). Then set up to heat over a very small flame, well guarded against wind. I use a luminous gas flame about 6 or 7 mm. high, coming from a burner tube of 3 mm. outside diameter. Continue heating at least one minute after the pool of acid has become clear white. The entire boiling and oxidation occupies about six to ten minutes.

As soon as the glass is cool enough to bear water, add sufficient ammonia-free water to dissolve the contents, and rinse out the solution quantitatively into a 50-cc. volumetric flask, in which it may be nesslerized.

B. THE METHOD BY ALIQUOT FRACTIONS: This method, alternative to the micro-oxidation just described, is less expeditious and convenient, but may perhaps find a wider variety of applications.

Procedure. Weigh out or measure a sample of the substance containing not less than 7 mgm. of nitrogen. In urine analysis, the sample will usually be 1 or 2 cc. Place this in a small flask of the typical Kjeldahl form with a capacity of 25-50 cc. exclusive of the neck. Add 1 cc. of sulphuric acid, 1 gram of potassium sulphate, and 3 drops of saturated mercuric chloride. Oxidize over a small flame taking pains that no froth rises into the neck of the flask. The oxidation may be expected to last from fifteen to twenty minutes. The resulting mixture is taken up in water, transferred quantitatively to a 100-cc. volumetric flask, filled to the mark, and mixed. An aliquot part, not more than 5 cc., and estimated to contain between 0.35 mgm. and 0.7 mgm., is taken out and placed in a 50-cc. volumetric flask, for the colorimetric test.

II. The Nessler color reaction.

In this reaction a strongly colored reddish or orange brown solution is produced by the action of ammonia upon mercuric potassium iodide in alkaline solution. The resulting solution must be perfectly clear, free from opalescence, showing a color intensity proportional to the amount of ammonia present. A deficiency of potassium iodide, or the presence of an excess of alkali or mineral

salts will increase the tendency to produce a cloudy solution. On the other hand, deficiency of alkali or undue excess of potassium iodide will interfere with the generation of the color.

It has been found that Winkler's solution can be altered so as to diminish the tendency to form precipitates, without interfering with a reliable development of the desired color. Among a considerable number of modifications of Winkler's formula which I have tested, the one here given seems best to meet the needs:

	grams
Mercuric iodide.....	15
Potassium iodide.....	10
Sodium hydroxide.....	40
Ammonia-free water to make.....	500 cc.

The sodium hydroxide is dissolved in part of the water, and set aside to cool. The two iodides are weighed out, mixed, and dissolved in about 15 cc. of water. They are then transferred with the aid of the alkali into a 500-cc. volumetric flask, and the whole is made up with water to the 500-cc. mark. Transfer to a stoppered Erlenmeyer flask, and allow to settle twenty-four hours before using. Or instead, if necessary, filter through a paper supported on a platinum cone. The paper must be tested in advance to be sure that it does not give a brownish color reaction with the reagent. This reagent is suitable without previous dilution to use with ammonia in any concentration from 0 to 1 mgm. per 50 cc. It is relatively tolerant of the presence of mineral salts in the mixture, and it can be mixed with water in nearly any proportions without risk of developing a precipitate within the first hour or two.

Procedure to Nesslerize. The 50 cc. volumetric flask containing the oxidized unknown is filled to about 40 cc. with ammonia-free water. A standard, containing 0.5 mgm. of nitrogen in the form of ammonium sulphate purified according to Folin,² is also placed in a 50-cc. flask and filled up to 40 cc. Into each of these flasks inject 5 cc. of the modified Winkler solution in a vigorous stream from a pipette. Fill to the mark, and stir thoroughly. The samples are ready for the colorimeter almost immediately.

Procedure for colorimetric test. The test should be carried out without undue delay, and in any case should be finished within the

² *Loc. cit.*, p. 496.

first hour. If the solutions are left for several hours, a turbidity is liable to develop, and during the incipient stages of this change it is possible to get totally incorrect colorimeter readings.

The color is satisfactory for determinations with about 20–30 mm. depth of liquid.

The reading of the standard divided by the reading of the unknown is equal to twice the nitrogen content of the nesslerized sample expressed in milligrams.

Any good colorimeter may be used, the Duboscq pattern being perhaps the most desirable. But if the substances being tested contain very little nitrogen, the cups in this style of instrument are too shallow.

The Autenrieth-Koenigsberger wedge colorimeter was used in the tests reported in this paper. It gives satisfactory results with the concentrations found in urines, but is inconvenient because of the difficulty in cleaning the wedge, and also because the range at the thick end of the wedge is too limited.

III. Accuracy of the method.

The two most important questions as regards accuracy are, first, whether the oxidation methods are reliable, and second, whether we can depend upon the results of nesslerizing under the given conditions. The following tests were carried out to verify these two points.

Samples were prepared containing: (a) 1 mgm. N as $(\text{NH}_4)_2\text{SO}_4$; (b) 0.5 mgm. N as $(\text{NH}_4)_2\text{SO}_4$; (c) 0.5 mgm. N as $(\text{NH}_4)_2\text{SO}_4$ + 0.5 cc. of the oxidizing mixture.

Each was nesslerized in 50 cc. Sample (a) was used to fill the wedge of the Autenrieth-Koenigsberger colorimeter, and was read successively against (b) and (c) in the capsule, the latter having a constant thickness of 100 arbitrary units.

	Reading of (a) against (b)		Reading of (a) against (c)
	50.0		49.00
	50.0		50.00
	49.5		49.50
	49.0		48.50
Average reading.....	49.6	Average reading.....	49.25
Scale correction.....	+0.5	Scale correction.....	+0.50
	50.1		49.75
Required.....	50.0	Required.....	50.00

546 Colorimetric Determination of Nitrogen

Sample (d), 2.5 mgm. commercial glucose, in 0.5 cc. To this was added 0.5 cc. of oxidizing mixture, and the oxidation carried out by the microchemical method. Oxidation was more difficult than for urines, but was complete in 15 minutes. Sample was nesslerized in 50 cc., and compared directly with known samples containing respectively 0.008 and 0.004 mgm. Unknown was between these two values, apparently about 0.006 or 0.007 mgm.

Sample (e), glucose as in (d) + 0.5 mgm. N as $(\text{NH}_4)_2\text{SO}_4$, oxidized microchemically, the same as (d).

Sample (f) 0.5 mgm. N, nesslerized directly (standard).

Sample (g) 0.5 mgm. N + 0.5 cc. oxidizing mixture, nesslerized directly.

Placed (f) in the wedge, and estimated against the others.

	(f) against (e)		(f) against (g)
	103.0		98.7
	103.5		99.5
	102.0		100.0
	101.0		100.0
Average reading.....	102.4	Average reading.....	99.6
Scale correction.....	0	Scale correction.....	0
	102.4		99.6
		Required.....	100

$$N = \frac{102.4}{100} \times 0.5 = 0.512$$

Required 0.506 or 0.507 mgm.

Sample (h), urine, sp. gr., 1.027 (uncorrected); analyzed as follows:

(h₁) Diluted to one-tenth with the oxidizing mixture, and 0.5 cc. (= 0.05 cc. urine) taken for analysis by the microchemical method.

(h₂) 1 cc. treated by the second method of oxidation, and a 5 cc. aliquot out of the 100 cc. (= 0.05 cc. urine) used for the colorimeter test.

(h₃) and (h₄), 5-cc. samples, analyzed by the Kjeldahl method.

	h ₁		h ₂
	During first half-hour	One hour after nesslerizing	During first 15 minutes
Readings..... (0.5 mgm. standard reads 100.)	78.0	78.5	77.5
	78.0	77.5	78.5
	78.3	78.0	77.7
	78.0	78.0	78.0
Average.....	78.1	78.0	77.9
Scale correction.....	+0.2	+0.2	+0.2
Corrected reading.....	78.3	78.2	78.1
Mgms. N in sample ($\frac{100}{\text{reading}} \times 0.5$).....	0.639		0.640

h₃. $\frac{N}{16}$ H₂SO₄ required = 46.1 cc. = 64.6 mgm. N.

h₄. $\frac{N}{16}$ H₂SO₄ required = 45.7 cc. = 64.0 mgm. N.

It appears from these tests that

1. The color produced by the modified Nessler reagent is strictly proportional to the quantity of ammonium salts, and is not measurably influenced by the presence of the oxidizing reagents. (See samples (a), (b), (c), (f), and (g)).

2. The relative intensity of unknown and known solutions remains constant amply long enough to allow good determinations (sample (h)). (Solutions containing less nitrogen will last much longer than this.)

3. The micro-oxidation method here described gives accurate results with known solutions that are more difficultly oxidizable than ordinary urines. (Samples (d), (e), (f), and (g)).

4. Urine analyses by both the methods described in this paper agree well with each other, and with the figures from the Kjeldahl method. (Sample (h)).

5. In conclusion, we may sum up, that it has been found possible to improve and abbreviate the Folin-Farmer colorimetric nitrogen method by avoiding the necessity of aspirating the products of oxidation. The resulting method is expeditious, reliable and pre-eminently simple. It requires less apparatus than the methods now in use, and we believe it is subject to fewer sources of accidental error.

CORRECTION

On page 236 of this volume, tenth line from the top, *Experiment XXXIV* should read *Experiment XXXIX*.

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